Toward Advanced Modular Drug and Gene Delivery System

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

July 2010

...to Kanar and my daughter Sarah

Acknowledgments

I am heartily thankful and truly indebted to my supervisor, Professor Cameron Alexander, for all his guidance, motivation and support during my studies. I would like to gratefully thank Professor Steven Howdle for helpful discussions and assistance. I gratefully thank Dr Snow Stolnik for all the helpful discussion, support and collaboration on the in vitro studies. I would particularly like to thank Dr Wenxin Wang for generously sharing his knowledge in polymer synthesis. I would like to thank Dr. Giuseppe Mantovani for helpful discussions and assistance. I would like to thank Dr Kris Thurecht for his help with RAFT agent synthesis.

Many thanks go to our lab technicians and especially to Christy for her help with my experimental work and her valuable assistance in solving everyday problems in the lab.

I acknowledge my coworkers in the advanced drug delivery group especially Dr. George Pasparakis, Johannes Magnusson, Felicity, Mahmoud and Sabrina for their useful discussions and company, without them the lab would have been a lonelier place.

I gratefully thank Mrs. Sheila Horseman for her invaluable assistance and kind support during writing up of my thesis.

I would like to thank Kurdish regional government and the University of Nottingham for financial support.

Most importantly I would like to thank my parents, my wonderful wife Kanar, lovely daughter Sarah and my father-in-law Mr. Sirwan for all their love and support.

List of Publications

In Refereed journals

1- Johannes Pall Magnusson, Aram Omer Saeed, Francisco Fernández-Trillo, S. Bersani, S. Salmaso, P. Caliceti and Cameron Alexander, Aqueous synthesis of "Smart" protein polymer conjugates with controllable architecture and substrate specificity. *(In preparation).*

2- Johannes Pall Magnusson, Aram Omer Saeed and Cameron Alexander, Synthetic Polymers for Biopharmaceutical Delivery, Polymer chemistry review. *(In preparation)*

3- Aram Omer Saeed, Johannes P Magnusson, Emilia Moradi, Mahmoud Soliman, Wenxin Wang, Snow Stolnik, Steven M. Howdle and Cameron Alexander, Multifunctional Bioresponsive Cell-Targeted Block Co-polymer Nanoparticles for Gene Delivery. *(Submitted-Journal of Bioconjugate Chemistry)*

4- Felicity Heath, Aram Omer Saeed, Sivanand S. Pennadam, Kristofer J. Thurecht and Cameron Alexander, Isothermal' phase transitions and supramolecular architecture changes in thermorresponsive polymers via acid-labile side-chains. *(Accepted-Journal of Polymer Chemistry)*

5- Yixiao Dong, Paul Gunning, Hongliang Cao, Asha Mathew, Ben Newland, Aram Omer Saeed, Johannes Pall Magnusson, Cameron Alexander, Hongyun Tai, Abhay Pandit[a] and Wenxin Wang, Dual Stimuli Responsive PEG Based Hyperbranched Polymers. *Polymer Chemistry., 2010, DOI: 10.1039/c0py00101e (Published)*

6- S. R. Abulateefeh, Aram Omer Saeed, J. W. Aylott, W. C. Chan, M. C. Garnett, B. R. Saunders and C.Alexander, Facile synthesis of responsive nanoparticles with reversible, tunable and rapid thermal transitions from biocompatible constituents, *Chem. Commun., 2009, DOI: 10.1039/b911986h.(Published)*

7- Aram Omer Saeed, Sabrina Dey, Steven M. Howdle, Kris Thurecht and Cameron Alexander, One pot controlled synthesis of biodegradable and biocompatible co-polymer micelles, *Journal of Materials Chemistry 2009, 19, (26), 4529-4535. (Published).*

8- Aram Omar Saeed, Johannes Pall Magnusson, Beverley Twaites and Cameron Alexander, "Stimuli-Responsive and 'Active' Polymers in Drug Delivery" in "Biomedical Applications of Electro active Polymer Actuators" *Carpi and Smela (Eds), Wiley 2009 (Published)*

9- Johannes Pall Magnusson, Adnan Khan, George Pasparakis, Aram Omer Saeed, Wenxin Wang and Cameron Alexander, Isothermal" Responsive Polymers Prepared in Water, *Journal of the American Chemical Society 2008, 130, (33), 10852 (Published)*

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Lists of Abbreviations

AAV	Adeno-associated viruses
AIBN	Azobisisobutyronitrile
APCs	Antigen presenting cells
ATRP	Atom Transfer Radical Polymerisation
BSA	Bovine Serum Albumin
BSTSE	2-(benzylsulfanylthiocarbonylsulfanyl) ethanol
CAC	Critical association concentration
cDNA	Calf Thymus DNA
CF	5-6-Carboxyfluorescein
CMC	Critical Micelle Concentrations
CuBr2	Copper (II) Bromide
DAEMA	Dimethylaminoethyl methacrylate
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DP	Degree of Polymerisation
E.E.	Encapsulation efficiency
EDC	1-ethyl-3-[3-dimethylaminopropyl]carbodiimide
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FR	Folate receptors
FT-IR	Fourier Transformed Infrared Spectroscopy
GI	Gastrointestinal
GPC	Gel Permeation Chromatography
GSH	Glutathione

GSH-MEE	Glutathione mono-ethyl ester
HBSS	Hank's Buffered Salt Solution
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPMC	2-hydroxy-propylmethylcellulose
I.M.	Intramuscular
I.V.	Intravenous
LRP	Living Radical Polymerisation
mPEG	methoxy Poly Ethylene Glycol
MPS	Mononuclear phagocyte System
MS	Mass spectrum
MWCO	Molecular Weight Cut Off
NMR	Nuclear Magnetic Resonance spectroscopy
NP	Nanoparticles
PBS	Phosphate Buffer Saline
PCL	Poly(□-caprolactone)
PDI	Polydispersity Index
PEGMA	Poly(ethylene glycol methacrylate)
PEI	Polyethylenimine
PEO	Poly(ethyle oxide)
PGA	poly(glycolic acid)
PLA	Poly(lactic acid)
PLGA	Poly(Lactic-co-glycolic acid)
PLGA	poly (lactide-co-glycolide
PLL	Poly-L-lysine
PMDTA	Pentamethyldiethylenetriamine
PPO	Poly(propylene oxide)
PVA	Poly vinyl alcohol
RAFT	Reversible Addition Fragmentation Transfer
RES	Reticuloendothelial system
RNA	Ribonucleic acid

ROP	Ring opening polymerisation
RT	Room Temperature
TEER	Trans-epithelial electrical resistance
TEG	Tetraethylene glycol
TEM	Transmission Electron Microscopy
THF	Tetrahydrofuran
UV/Vis	Ultra Violet/Visual spectroscopy
W/O	Water in Oil (emulsion)
W/O/W	Water in Oil in Water (emulsion)

Abstract

In chapter two, the development of new a nanoparticulate carrier system for gene delivery was described. The new nanocarrier consists of a blend matrix formed by a poly (lactic-co-glycolic acid) (PLGA) and Poly (ethylene glycol) bis (3-aminopropyl) terminated (also known as JeffamineTM). Nanoparticles were formulated based on a 50:50 weight ratio of PLGA: Jeffamine using a modified emulsification-solvent diffusion technique. The potential of these blended matrix nanoparticles for encapsulation efficiency of Calf Thymus DNA and release profile were also studied. The achieved encapsulation efficiency of Calf Thymus DNA was approximately 84% for 0.4% theoretical loading with regard to total amount of PLGA. The PLGA: Jeffamine blended nanoparticles provided continuous and controlled release of Calf Thymus DNA. The PLGA: Jeffamine nanoparticles were also coated with PLGA-PEGMA$_{475}$ and PDMAEMA-PEGMA block copolymers using a simple physical adsorption method. After surface coating of the nanoparticles, zeta potential value showed significant reduction of surface charges from -38 mV to near zero value, while TEM micrographs showed a well defined core-shell nanoparticle.

In chapter three, A facile route to biocompatible poly (lactic acid-co-glycolic acid)-co-poly (ethyleneglycol methacrylate) (PLGA-PEGMA) block co-polymers was described utilising a combination of ring-opening polymerisation (ROP) and Radical Addition Fragmentation Transfer (RAFT) methods. A series of PLGA-PEGMA polymers varying in co-monomer content and block length were synthesised with low polydispersities. All the block co-polymers formed micelles in aqueous solution as shown by dynamic light scattering, while critical micelle concentrations were found to be in the micromolar range.

The polymer micelles were able to encapsulate model drugs (carboxyfluorescein and fluorescein isothiocyanate) and selected co-polymer micelles incubated with 3T3 fibroblasts as a model cell line were rapidly taken up as indicated by fluorescence microscopy assays.
The combination of the polymer chemistries opens the way to highly flexible syntheses of micellar drug carrier systems.

In chapter four, multifunctional and modular block co-polymers prepared from biocompatible monomers and linked by a bioreducible disulfide linkage have been prepared using a combination of ring-opening and atom-transfer radical polymerizations (ATRP). The presence of terminal functionality via ATRP allowed cell-targeting folic acid groups to be attached in a controllable manner, while the block co-polymer architecture enabled well-defined nanoparticles to be prepared by a water-oil-water double emulsion procedure to encapsulate DNA with high efficiency. Gene delivery assays in a Calu-3 cell line indicated specific folate-receptor-mediated uptake of the nanoparticles, and triggered release of the DNA payload via cleavage of the disulfide link resulted in enhanced transgene expression compared to non-bioreducible analogues. These materials offer a promising and generic means to deliver a wide variety of therapeutic payloads to cells in a selective and tuneable way.

Chapter 1

Polymeric drug and gene delivery systems

1. Introduction

The efficacy of synthetic drug compounds in therapy is strongly dependent on their formulation into medicines and on their distribution, localisation and accumulation in different regions in the body. The ability to deliver a drug compound to the specific target site (organ, tissue, cell, intracellular compartment)s remains a challenge. A century on from Paul Ehrlich's visionary hypothesis, for which he received the Nobel Prize in 1908, there are still no 'Magic Bullets'[1] against most diseases. As a result, the delivery of drugs to the desired target sites in the body at the right time, and in the right dose is still an unmet clinical need[2]. This leads to inefficient use of drugs, undesired side effects and greater medical intervention, with resulting burdens on patients, carer populations and healthcare budgets. Current targeting methods for many drugs either lack specificity or are not active for certain patient groups. As a consequence, drug delivery systems are gaining in importance - and many of these are based on polymers[2, 3]. In addition, new generations of therapeutics are emerging, and these to are often macromolecular or polymeric in nature. For example, in 2002 and 2003 the US FDA approved more biotechnology products (proteins and antibodies) and drug delivery systems as marketed products than new low

molecular weight drugs[4]. However, these new classes of drugs and their conjugates, complexes and formulated vehicles - sometimes considered as Nanomedicines - and the related biotherapeutics, still urgently require technologies to ensure their specific localisation in target sites[5, 6]. As a consequence, new modalities of 'smart' or active materials ranging from bioresponsive to electroactive polymer drug delivery systems are being developed[7, 8]. In this chapter, a short introduction is given to key concepts in drug delivery, considering the specific issues for polymers and smart materials related to the transport and release of therapeutic compounds, and highlighting some exciting recent examples where polymers have been developed to help target and deliver drug molecules. The focus is primarily on soluble polymers and nanoparticles rather than hydrogels, as there are numerous reviews on responsive hydrogels available[9-19], and the design concepts in the soluble and nanoparticle polymer systems best exemplify the key responses that can be engineered into 'smart' materials.

1.1. Drug Delivery - Examples, Challenges And Opportunities For Polymers

In general, drug delivery aims at optimising therapy by delivering bioactive agents at specific sites or specific rates to the patient[20]. The field has evolved from simple topical waxes[21] and delayed release formulations to the targeted delivery of therapeutic agents to specific cells and subcellular compartments[22]. Traditional approaches to drug delivery have been based on simple formulation parameters. For low molar mass compounds (Mw < 1000), which still represent the main focus for the pharmaceutical industry, the goal is to deliver the drug to the pharmacological receptor or biochemical target with as little

complexity in the formulation as possible. Since many of these compounds are relatively lipophilic and can partition across membranes, the drug delivery system needs to address the biopharmaceutical requirements of the dosing route, and these involve a variety of physical and biochemical barriers. Delivery is ultimately dependent both on the physicochemical properties of the drug and on the physiology of the body, thus a brief consideration of these factors and the common dosing routes is needed to put polymer-mediated drug delivery in context.

1.1.1. Oral Drug Delivery Systems

The oral route is the most widely used for drug administration and is likely to remain the most favoured route in the future owing to good patient compliance, familiarity and a long history of successful development in the pharmaceutical industry[23, 24]. However, as many candidate therapeutics in development are increasingly of higher molar mass and poorer aqueous solubility compared to existing drugs, the systems for formulating and delivering these candidates are becoming more complex. For conventional oral delivery water-soluble drugs can be formulated into tablets or capsules with controllable erosion or breakdown profiles to determine rate and site of drug release in the GI tract[25]. The types of polymers used in these systems are relatively simple, and include cellulose and derivatives e.g. 2-hydroxy-propylmethylcellulose (HPMC) and sodium carboxymethylcellulose, which are often combined with other materials such as poly(N-vinylpyrollidone) and poly(methacrylic acid/methacrylates) for optimal formulation properties[26]. There are a great number of potential variations possible in these drug delivery materials, and many have been successfully commercialised.

However, in the case of many poorly-soluble drugs and for targeting specific sites in the body, current matrices do not exhibit the right balance of functional behaviour. For the new candidate drugs, polymers that have one set of properties in the oral cavity and another type of behaviour in designated areas of the GI tract may offer an answer to the delivery problem. Drugs of poor aqueous solubility can be formulated or conjugated to polymers that are soluble at high pH, but hydrolyse at low pH to enable drug transport from the oral cavity to the stomach[25]. The change in polymer properties can be in response to the local environment or induced by external stimuli such as charge, pH, light and temperature[27, 28].

1.1.2. Parenteral Drug Delivery

For parenteral drug delivery, e.g. intravenous (i.v.) or intramuscular (i.m.) injection, polymers can be used to enhance solubility, either directly, or through the formation of higher order systems, such as micelles which can carry a water-insoluble drug molecule in their hydrophobic cores[28, 29]. Polymers can also be used for parenteral drug delivery in the form of micro- and nano-particles[30], as long as they meet the necessary criteria for injectable formulations of being sterilisable and stable against aggregation. The latter point is particularly important as material injected into the blood stream rapidly passes into the heart and then round the pulmonary circulation - any particles larger than ~ 5 μm can lodge in pulmonary capillaries resulting in embolism[31]. Thus, even for relatively simple polymers in parenteral use, careful consideration must be given to biopharmaceutics and formulation requirements - for more complex responsive polymers the properties in the *in vivo* environment must be very tightly defined in order to prevent unintended adverse

reactions. Nevertheless, the potential advantages of using 'smart' nanoparticles for parenteral delivery remain, as it can be envisaged that a polymer surface coating that enables prolonged circulation in the bloodstream, but which can be actuated to expose a binding moiety in response to a biological or external signal. Targeting in this case could be direct and instantaneous. Another facet of parenteral drug delivery is the implanted device, and this is perhaps the most promising and most readily commercialised area for responsive and/or active polymers[32-34]. For an implanted vehicle or depot, drug release rate is controlled by dissolution and/or diffusion in the formulation, or for solid polymer implants by diffusion and/or degradation of the polymer. For more complex polymer hydrogels[35], the release can be controllable by the linking chemistries, and these can be made responsive to a wide variety of stimuli such as enzymatic action, redox potential as well as those noted above for the oral route.

1.1.3. Topical and Transdermal Drug Delivery

For other routes of delivery, the formulation considerations relating to polymers are less restrictive than for the parenteral route. Typically, for local rather than systemic activity, polymers can be used to enhance the efficacy of topical drug delivery systems through solubility enhancement and/or skin hydration[36]. For transdermal delivery, polymers can have several roles, either as solubility modifiers, permeation enhancers[37] or as aids in iontophoresis. The presence of multiple-charged species is a prerequisite for iontophoretic movement of drug molecules through the skin, so can be considered as an actuation process in its own right. However, clinically, iontophoresis is still a niche area in drug delivery and

other routes are currently more attractive for development of responsive polymer systems, although some examples have been developed[38, 39].

1.2. Gene therapy

The ability to manipulate genes and gene expression has tremendous therapeutic potential in the treatment of many of inherited and acquired diseases. Advances in molecular biology and human genome projects have provided important information on genetic control of cellular processes and disease pathogenesis[40]. Therefore, identification of genes involved in disease and cellular processes can be novel targeted in therapeutic approaches for the treament of many inherited and acquired diseases via manipulation of targeted genes in diseased cell populations[41, 42].

The purpose of gene therapy is to manipulate cellular processes by altering gene expression, in specific cell populations via delivery of DNA, RNA, or antisense sequences. Originally, gene therapy was designed to treat inherited genetic disorders such as cystic fibrosis and haemophilia, but recently the applications of gene therapy have also expanded for acquired disease conditions such as HIV, cancer and wound healing[43-45].

In the first example of inherited disorders, the cellular process can be restored via replacing the deficient genes, whilst acquired diseases such as HIV can be treated by changing the expression of a gene to stimulate desirable cellular events such as stimulation of immune response[46, 47].

Generally, gene therapy and manipulation of genes can be carried out in two different approaches, either *in vivo* or *ex vivo*. The *ex vivo* approach includes isolation of cells from the patient, genetic modification of the cells and finally implantation of them back into the patient. The advantage of this approach is high transfection efficiency, but the procedure is very costly and can not be done in all cases[48].

The second approach in gene therapy is *in vivo*, in this case the therapeutic gene is inserted into the patient using a gene delivery system (vector), this being more challenging and requiring many variables to be controlled in order to build-up a safe and efficient gene delivery system. There are two types of vectors to use in *in vivo* gene therapy, viral vectors and non-viral vectors[49].

Viral vectors are considered to be the most efficient delivery vector owing to their known ability to effectively pass the biological barriers and reach cells. The design of viral gene delivery system involves removal of a section of the genome to prevent replication and encoding a therapeutic gene into their genome.

Viral vectors, despite their ability to deliver therapeutic genes efficiently, have drawbacks and limitations in their applications. The major hurdles in using viral vectors as gene delivery vectors are: limited space to insert genes in their genome, induction of immune response against the viral proteins, possible recombination with wild-type viruses, difficulties experienced in large-scale pharmaceutical productions[50, 51]. Therefore, interest in introducing non-viral vectors has hugely increased.

Non-viral approaches involve either plasmid DNA or oligonucleotides which are generally considered to be safe. However, comparing non-viral gene delivery vector to viral gene vectors, they are significantly less efficient and this has remained as difficult challenge to overcome and improve the therapeutic applications.

1.2.1. Nucleic Acids delivery and challenges for in vivo gene therapy

The use of nucleic acids in therapy involves many hurdles, including difficulties in formulating the delicate biopolymer drug and the high chances for degradation en route to the target. For DNA-based therapies the target is the cell nucleus[52, 51], whereas for RNA, delivery to the cytoplasm is required[53]. Many designed polymeric nucleic acid delivery systems have been developed by analogy with viruses vectors. The requirements for these vectors are stringent: for example, for DNA, the nucleic acid (which can be several million Da in molar mass) must be condensed to a small size, and protected from serum and intracellular nucleases. The DNA must be delivered to the target cell, cross the external cell membranes passively or actively, leave the endosomal compartments (avoiding degradative enzymes and escaping traffic to the lysosomes), then it must translocate into the nuclear compartment ready for transcription. The vector must thus protect the DNA and help transport it across multiple cell barriers, yet release it at the correct location. In addition, for an injectable DNA delivery formulation, the vectors with encapsulated/complexed DNA must be capable of extended circulation in the bloodstream in order to have a chance to reach their cellular target. They must also be small enough to gain access to tissues and cells - typically this will put a size limit of <250 nm for

the hydrodynamic diameter of the vector system in circulation. For commercial development, it is also desirable for a gene delivery vector to have flexible tropisms, so that one vector can be adapted for application across a wide range of disease targets. Above all, the vectors must be safe, i.e. non-toxic, non-immunogenic and fully cleared from the body after use. It is the safety factor which has hampered the development of viral DNA and RNA delivery systems to date, as although viruses have evolved to be very successful in overcoming cell barriers, their therapeutic use is dependent on deactivation of their infective properties. Immune reactions are also a major problem that needs to be solved for longer term use of viral vectors in nucleic acid delivery. A fuller discussion of viral gene therapy is beyond the scope of this thesis[54], but the safety issues associated with viruses have prompted intense research into synthetic viral mimics. Transfection efficiency, acute and long-term toxicity and *in vivo* fate are all important challenges for synthetic polymer vectors as well as viral systems. Considering all the demanding requirements it is perhaps not too surprising that there are no successfully commercialised synthetic gene delivery vectors in the clinic so far. Described later in this chapter are some of the more specific challenges facing polymeric gene delivery vectors, but it should be noted that there are still a large number of these systems in the research and development stages, most likely as a result of the change in treatment paradigms that will occur should successful gene therapies reach the clinic.

To achieve efficient gene delivery, the naked DNA has to pass through extra-cellular barriers and become internalised by the desired cell population.

Because of the existence of many barriers extra-cellularly and inter-cellularly the efficiency of naked DNA delivery is significantly hampered.

The main hurdles are the presence of extra-cellular endonucleases, enzymes with the ability to degrade naked DNA within 30 minutes[55]. The extensive uptake by scavenger receptors on the liver also leads to rapid clearance of DNA from the blood circulation and this prevents the distribution into different organs and tissues[56, 57].

Furthermore, because of its size and surface charges, DNA is unable to pass across the cell and nuclear membranes. The plasmid DNA is typically 10^3 to 10^4 base pairs in length (converting this into lenght distance for a stretched DNA of 10^4 base pairs multiplied by 0.34nm base pair distance results in a total lenght of 3.4μm), has a supercoiled tertiary structure in aqueous solutions, a molecular weight of 10^6-10^7-Da and an effective hydrodynamic diameter of greater than 100nm[49]. The surface charge density of naked DNA, which has zeta potentials ranging from -30 to -70 mV, creates repulsion between the DNA and the negatively charged cell surface. This negative surface charge density most likely limits the uptake of DNA by cells.

Therefore, all these hurdles and barriers have inspired the development of gene delivery systems that can protect the DNA from degradation and at the same time transport the DNA through extra and intracellular barrieres efficiently to reach to nuclei and to exert their therapeutic effect.

A number of mechanical techniques have been used to enhance the delivery of naked DNA, among these techniques are gene gun, hydrostatic pressure, electroporation, continuous infusion, and sonication[58-61].

1.2.2. Nonviral gene delivery systems

1.2.2.1. Cationic based gene delivery systems

There are two different types of non-viral gene delivery systems, cationic lipid[62, 63] and cationic polymer delivery systems[64, 65]. Both cationic lipids and polymers are capable of condensing DNA via charge-charge interactions which lead to reduction in size and surface negative charge. In addition to reduction in size and negative surface charge, they can also protect the DNA from degradation by nuclease enzymes.

1.2.2.1.1. Cationic lipid

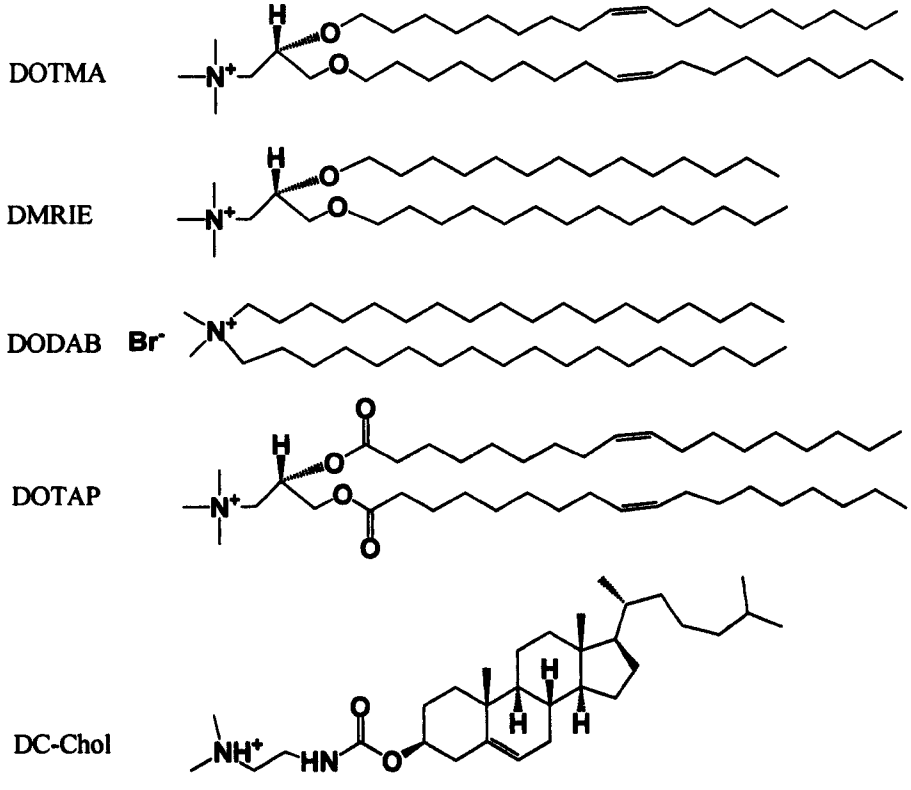
Cationic lipids to condense DNA were first used by Felgner *et al*[66]. Cationic lipids structures are composed of several parts, including a hydrophobic segment, a linker and a positively charged end-group. The chemical compositions of cationic lipids are mainly fatty acids (derived from oleic and myristic acid) or cholestrol groups in combination with a charged component[67].

Different parts of cationic lipid structures provide different functionalities, for example, the hydrophobic segment mimic the physical properties of lipid bilayers. The linker is responsible for biodegradablity and more importantly the positively charged end-group is responsible for interaction with the negatively charged phosphate groups on the DNA backbone, leading to condensation and packaging of DNA into smaller size[68, 69].

The structure of the most widely studied cationic lipids as gene delivery systems are summarised in Figure 1-1.

There are a number of cationic lipid based formulations that have been used in many *in vitro* transfection of DNA and some of them are commercially available. Examples of commerically available cationic lipids are Lipofectin® (a 1:1 mixture of DOTMA and DOPE), Transfectam®, LipofeACE™, LipofectAMINE™, and LipoTAXI™[70].

Cationic lipids



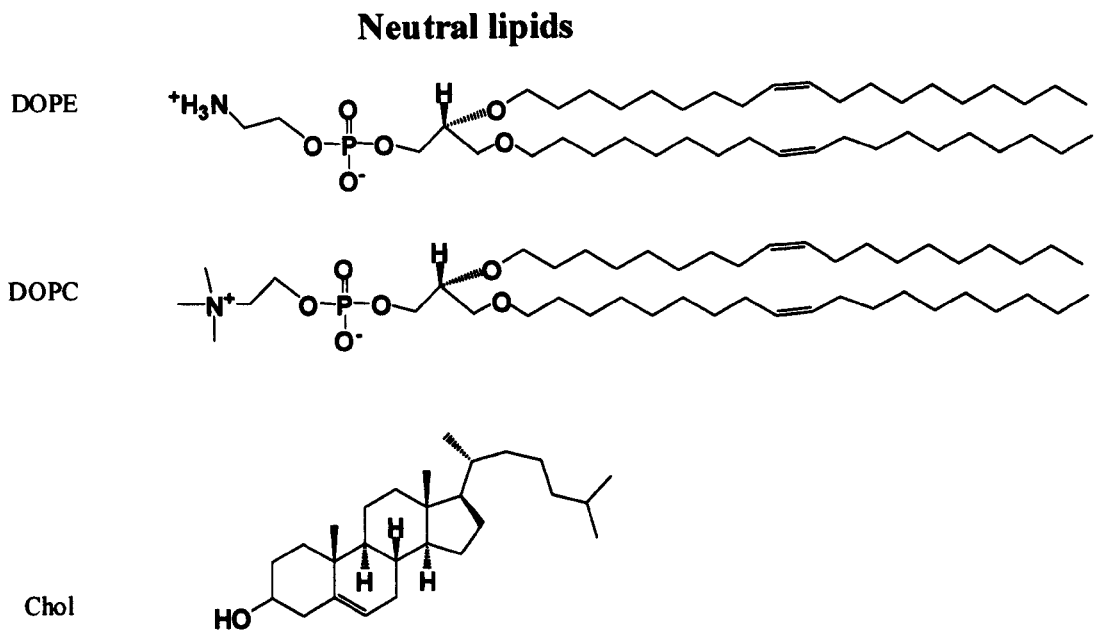


Figure 1-1. Structure of common lipids used as materials for gene therapy. Cationic lipids: DOTMA: (2,3-bis(oleoyl)propyl)- trimethyl) ammonium chloride; DMRIE: 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DODAB: dioctadecyldimethyl ammonium bromide; DOTAP: 1,2-diacyl-3-trimethylammonium propane; DC-Chol: 3[N-(N0,N0-dimethylaminoethane)- carbamoyl] cholesterol. Neutral Lipids: DOPE: dioleoylphosphatidyl ethanolamine; DOPC: dioleoylphosphatidyl choline; Chol: cholesterol.

Synthesis of several novel cationic lipids derivatives revealed that the transfection efficiency of these derivates varies by changing the headgroup or the length of the linker. It has been reported that multivalent headgroups, such as spermine, in a T-shape configuration tend to be more effective than their monovalent counterparts at facilitating gene transfer[71, 72, 67, 73, 69]. On the other hand, increase in gene delivery activity was enhanced by increasing the length of the linker[71].

Interaction of cationic lipids with DNA results in the formation of a condensed structure which has been termed a 'lipoplex', in which the DNA is hidden within the cationic lipid[74, 75]. The colloidal properties of the lipoplex such as stability and size are usually determined by the charge ratio (+/-) between the lipid/DNA[76]. The charge ratio (+/-) is the ratio between the number of amine groups on the cationic lipids in relative to phosphate groups on DNA backbone. The charge ratio (+/-) has a crucial effect on the packaging of DNA, as neutral charge (ratio of 1:1 lipid to DNA) is avoided because it results in formation of large aggregates[76]. It has also been noticed that the order of addition effect on the lipoplex size[74, 76], by adding DNA to the lipid, a gradual increase in size was noticed, whereas, adding cationic lipid to DNA did not change the particle size until the positive charge of the lipid exceeded the negative charges on the DNA backbone and at this point rapid increase in size were noticed[74].

The net charge on the lipoplex is important for *in vivo* and *in vitro* transfection owing to their interaction with other components (e.g. medias, serum, glycoproteins) which can hamper the transfection efficiency.

In the case of *in vitro* transfection studies, a positive charge ratio results in increasing the interaction with the cell membrane, and thus enhancement the transfection, whereas the net charge ratio for *in vivo* studies is more problematic owing to the presence of multivalent anionic species in serum, which can interact with the lipoplex and enhance the fusion of the lipid together, which lead to increasing the particles size[77]. Other components of serum such as large polyanions (e.g Heparin) can compete with DNA to bind with the cationic lipid and displace the DNA in the lipoplex[77].

To overcome this problem which is associated with the colloidal stability of the lipoplex due to charge neutralisation, incorporation of polyethyleneglycol phosphatidylethanolamine (PEG-PE) in to cationic lipids has been successful. PEG prevented the aggregation and interaction with the serum components and led to increased the stability of the lipoplex[78-80].

1.2.2.1.2. Cationic polymers

Synthetic cationic polymers and their derivatives for gene delivery include synthetic peptides[81, 82], poly-L-lysine (PLL)[83], polyamines (such as polyethylenimine (PEI)) and polyamidoamine dendrimers, and poly(vinylimidazole) derivatives[84-86]. These polycations self-assemble with DNA to form charge-neutralised or cationic complexes, dependent on charge ratio. Higher transfection efficiency and serum sensitivity have been achieved with polycation systems compared to lipoplexes[87]. Although gene transfer in some polycation vector systems, such as poly-L-lysine, is rather low compared to viral vectors, the use of the multiply-charged polyethylenimine (PEI) has led to an effective gene delivery in many cell lines. To date, PEI remains the most effective polymer for gene delivery. It has been shown to target DNA *in vivo* and *in vitro*, to promote nuclear targeting, and to facilitate DNA escape from endosomes[88]. The action of PEI in part depends on its pH-responsive character, but PEI homopolymer is toxic at levels required for high transfection, and thus other candidate polymers with pH or other responsive characteristics are required for medical use. An emerging area of research is the use of synthetic polymers that are able to bind DNA in a reversible manner employing functionality that bestows

polymer conformation and hydration changes as well as DNA recognition.

In order for non-viral systems to be effective vectors, the polymer must not only condense and deliver DNA intact to the target site, but must also enable nucleic acid to be transported through the cell membrane, and be translocated from the cytoplasm to the nucleus. Many researchers have now investigated DNA complexed by electrostatic interactions to cationic polymers as a method by which the therapeutic gene can be transported[89]. In most cases these cationic polymers form condensed complexes with DNA that both contract the nucleic acid to facilitate cellular uptake, and which protect it from serum and cytosolic nuclease degradation. Mechanisms of DNA condensation, cellular uptake and transport to the nucleus, as well as strategies to reduce toxicity, improve transfection potential and nuclear targeting of polycation-mediated delivery systems are discussed below.

1.2.2.2. Mechanisms Of Non-Viral Polycation-Mediated DNA Delivery

DNA condensation is a naturally occurring process *in vivo*, and is important for cellular processes, such as DNA replication and transcription. DNA condensation by polycations is of great importance if external DNA is to reach the nucleus of target cells. Although the DNA condensation is important during cell division, but would hinder DNA replication and transcription, during which it has to be unbound; DNA undergoes rounds of condensation and decondensation, depending on the phase of in the cell cycle. DNA is condensed by electrostatic neutralisation of the negative charges along the phosphate backbone by the positively charged polycation.

At a certain critical extent of charge neutralisation (around 90 % for polycations) DNA is condensed into small, tightly packed nanoparticles of between 20-200 nm diameter, through localised bending, resulting in the formation of a variety of condensed DNA structures, often reported to be toroid, rod-like, or spheroidal[90].

Indeed, it has been proposed by Pollard *et al* that nuclear trafficking of complexed DNA is dependent more on the spherical morphology of condensed DNA, rather than on the ionic interactions[91]. This work was confirmed by Liu *et al* who observed that small, spherical complexes were more efficient for receptor-mediated uptake[92]. As well as localised changes in DNA structure, factors such as reduction in DNA segment interactions, as result of polycation binding, and DNA-solvent interactions contribute to its condensation into various morphologies. Several molecules of plasmid DNA may become incorporated into the condensed structure, and thus it is often difficult to distinguish condensation from aggregation or precipitation. The size and morphology of condensed particles are dependent on many thermodynamic and kinetic factors[90].

The formation of these particles is crucial for entry through the cell membrane, protection from nucleases, and consequent transfection ability. The process of DNA condensation is reversible, and can be defined as the dramatic decrease in volume (10^4 fold) occupied by a DNA molecule, usually with a finite size, and orderly morphology. There has been much experimental and theoretical investigation into the organisation of DNA into its condensed state, and the formation and arrangement of compacted DNA structures, including toroids, rods, and intermediate structures. As DNA condenses, it undergoes a coil-globule transition. Detailed fluorescence studies indicate that rods and toroids are formed as a result of the coil-globule transition of DNA. Once a toroid has reached a certain critical size, it can no

longer package DNA in the usual way, and the remaining coil condenses into an irregular globular structure[93].

Many polymers are able to condense DNA to these toroidal and contracted nano/microsphere structures *in vitro* and the importance of efficient condensation for transfection has been demonstrated[94]. There are a number of factors that influence DNA condensation and the size of polymer-DNA complexes. For example, low molecular weight linear PEI forms much larger complexes with DNA than higher molecular weight and branched PEI, and these complexes possess lower transfection abilities.

1.3. Cellular Uptake Pathways in Nonviral Gene Delivery System

In general, the therapeutic effect of any drug is exerted upon reaching the desired site of action. If this does not occur, the drug will have no therapeutic activity or may produce adverse effects through interaction with undesirable targets. In the case of gene therapy, reaching the targeting site is far more complicated since large, charged DNA molecules are difficult to transport in the body[95]. For DNA therapy, the targeting site is nucleus. Cell membranes which amphiphilic in nature (hydrophilic on the outside), present a restricting barrier specially for large, hydrophilic and charged molecules. Therefore, a suitable gene delivery system is required to achieve an efficient cellular uptakes of such molecules.

These barriers which include binding to the cell surface, traversing the plasma membrane, escaping lysosomal degradation, and overcoming the nuclear envelope[96]. As described in section (1.2.2. chapter 1), the most commonly used materials in preparations of nonviral gene delivery systems include cationic lipids and cationic

polymers. Introduction of some functionalities onto these delivery systems can enhance the cellular uptake, such as using targeting ligands to increase cellular uptake through receptor-mediated endocytosis (endocytosis refers to a process of cellular uptake of macromolecules and solutes into membrane-bound vesicles derived by the invagination and pinching off of pieces of the plasma membrane)[95, 97], peptides to enhance endosomal escape[98] and nuclear localisation signals to enhance nuclear delivery[99]. Endocytosis can be classified into two categories, phagocytosis (the uptake of large particles) and pinocytosis (uptake of fluids and solutes)[100]. Phagocytosis also differs from pinocytosis in that it occurs only in mammalian cells, whereas pinocytosis occurs in all cells[101]. In addition, there are four morphologically different pinocytic pathways which include clathrin-mediated endocytosis, caveolae based endocytosis, macropinocytosis, and clathrin/caveolae-independent endocytosis[102]. The difference between these pinocytic pathways are composition of the coat, size of the detached vesicles and fate of internalised particles, (Figure 1-2).

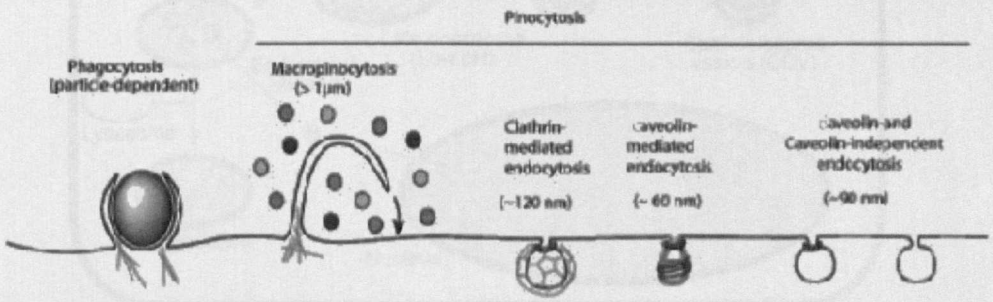


Figure 1-2. Endocytosis pathways in nonviral gene delivery systems, The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the molecules to be internalised (ligands, receptors and lipids) and the mechanism of vesicle formation[101].

The detailed mechanisms underlying the different uptake pathways are beyond the scope of this thesis, However, it is important to note that endocytosis pathways are considered to be the main mechanism for the internalisation of most nonviral gene delivery system into the cells[103]. The uptake mechanisms also directly or indirectly influence intracellular traffic pathways which dictate the fate of the vectors. Intracellular traffic refers to the process after internalisation in which the molecules tend to be trapped in intracellular vesicles which eventually fuse with lysosomes where they are degraded or released into the cytoplasm. Thefore, designing a suitable gene delivery system is required to increase the cellular uptake and improve the intracellular fate of the vector. A schematic of clathrin-mediated endocytosis as shown in the (Figure 1-3).

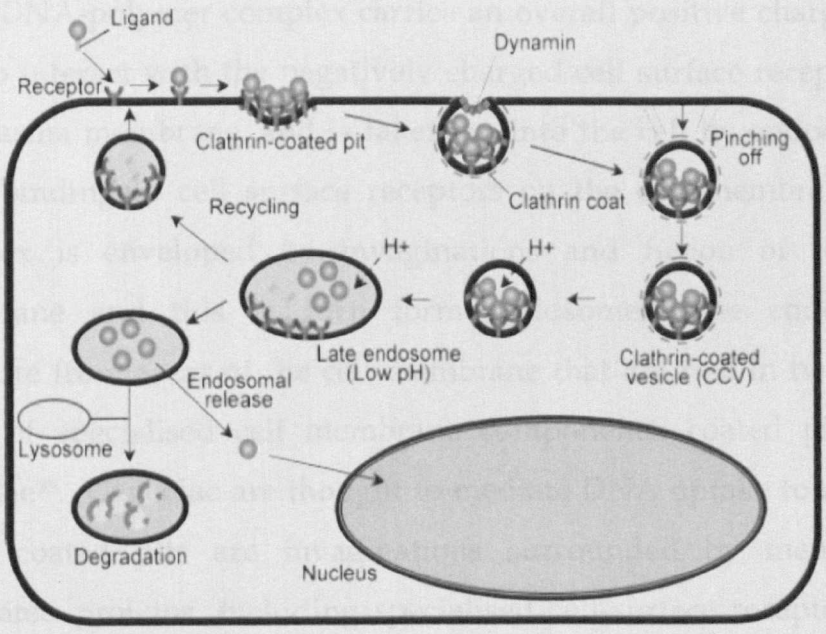


Figure 1-3. Receptor (clathrin)-mediated endocytosis[95]. The ligand binds to a specific cell surface receptor. This results in the clustering of the ligand-receptor complexes in coated pits on the plasma membrane.

The coated pits then invaginate and pinch off the plasma membrane, aided by dynamin, to form intracellular clathrin-coated vesicles. The clathrin coat then depolymerizes, an endosome forms. Molecules entering by this pathway experience a drop in pH from neutral to pH 5.9 to 6 in the lumen of early endosomes, with a further reduction to pH 5 during the progression from late endosomes to lysosomes. The low pH in endosomes causes the ligands to dissociate from the receptors and recycle back to the cell membrane. The vesicles then fuse with other late endosomes and eventually fuse with lysosomes in which the particles are degraded.

1.3.1. Cellular Uptake Of Non-Viral Polymer-DNA Complexes

If the DNA-polymer complex carries an overall positive charge, it is able to interact with the negatively charged cell surface receptors of the plasma membrane, and is taken up into the cell by endocytosis. After binding to cell surface receptors on the cell membrane, the complex is enveloped by invaginations and fusion of the cell membrane and this in turn form endosomes. The endosomes originate from areas of the cell membrane that are rich in two main types of specialised cell membrane components: coated pits and caveolae[95]. Caveolae are thought to mediate DNA uptake to muscle, while coated pits are invaginations surrounded by membrane-associated proteins, including specialised cell-surface receptors, for specific cellular uptake (receptor-mediated endocytosis). Once inside the membrane-bound endosome, any material (≥ 250 nm diameter) is usually processed by cellular mechanisms. Thus polymer-nucleic acid complexes must be strictly controlled in terms of their size to be therapeutically active[95].

Endosomes become fused with golgi hydrolytic vesicles to form endolysosomes, and ingested material is broken down by hydrolytic enzymes activated by the acidity (pH 5-6) inside the active lysosome. As proton ions are pumped into the endolysosomes, an influx of chloride ions occurs, which relieves the accumulated proton gradient. The osmolarity within the lysosome increases, as a result of the influx of chloride ions. The resulting influx of water causes the endo-lysosome to swell, and eventually burst, after degradation of ingested material.

The most common uptake pathway into the cell for non-targeted polymers occurs via passive endocytosis, and indeed has been shown to be the pathway for both PEI (polyethyleimine) and PLL (poly L-lysine)[104, 105]. It is also likely that transfection occurring via endocytosis takes place for many polymers through calcium-mediated cell anchorage to the extracellular matrix. The normal process of endocytosis and digestion by lysosomes, is potentially a barrier to non-viral delivery systems that rely on non-specific cellular mechanisms to gain entry into the cell. A non-viral vector must be able to avoid degradation by lysosomes if it is to access the nucleus and be transcribed. Cationic polymers such as PEI, are only partially protonated in the serum and cytosol, but become fully protonated at low pH within the endolysosome as they accept and buffer the proton ions that are pumped into the vesicle. The buffering action of PEI raises the pH within the endolysosome, resulting in inactivation of hydrolytic enzymes. In addition, the increased charge of protonated PEI results in an influx of chloride ions and water to counter the increase in osmotic pressure inside the endolysosomes and this can act to burst the membranes.

It is likely that PEI and other polymers complexed to DNA are released from the lysosome as it ruptures before the hydrolytic enzymes can become activated, and degrade the nucleic acid. The overall process by which PEI escapes through its protonation and endosomolysis is often termed the 'proton sponge' mechanism[106, 107].

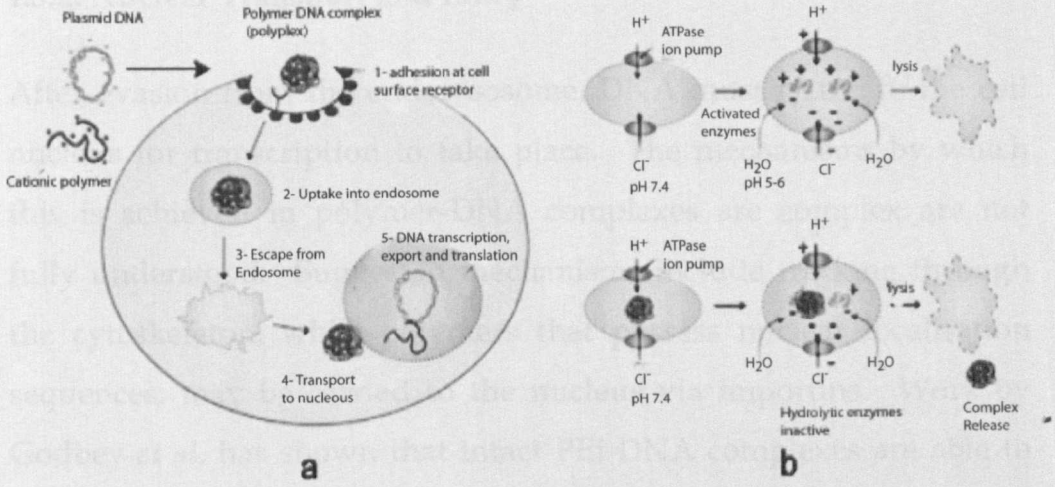


Figure 1-4. Schematic of cellular uptake of non-viral vector-DNA complexes, and evasion from lysosomal degradation. Diagram (a) shows the general proposed method, and diagram (b) illustrates more detailed mechanism of endolysosomal escape. In diagram (b), the top figures outlines normal lysosomal degradation through active hydrolytic enzymes. Due to the buffering capacity of many polycations, hydrolytic enzymes are not activated, and the DNA-polymer complex is able to escape degradation.

Transfection of polymer-DNA complexes usually requires actively dividing cells, and transfection efficiency is enhanced prior to mitosis, when the nuclear membrane has been broken down. Therefore gene therapy with non-viral cationic vectors is

advantageous in delivery to e.g. brain tumours, where normal cells do not divide and therefore are not transfected, but malignant cells undergo active cell division and are able to be targeted and destroyed. Brunner *et al*, demonstrated that linear PEI was not dependent on the cell cycle for transfection.[108, 109]

1.3.2. Nuclear Transport and Entry

After evasion from the endolysosome, DNA must traffic to the cell nucleus for transcription to take place. The mechanisms by which this is achieved in polymer-DNA complexes are complex are not fully understood. Suggested mechanisms include tracking through the cytoskeleton, while polymers that possess nuclear localisation sequences, may be carried to the nucleus via importins. Work by Godbey *et al*, has shown that intact PEI-DNA complexes are able to enter the nucleus as discrete particles (possibly vesicular in structure)[110, 111]. Both PEI and PEI-DNA were shown to enter the nucleus, with a suggested mechanism involving coating of complexes with a lipophilic layer, which then fuses with the nuclear membrane. This coating may arise from the remains of the membrane-bound endolysosome or through electrostatic binding of anionic phospholipids to the cationic complex. Both coating mechanisms have been shown to take place with cationic liposomes and it is not unreasonable that nuclear uptake occurs in the same manner for other cationic polymers.

For the design of polymer gene delivery vectors, it might be assumed that a mechanism must be encoded into the polymer to allow dissociation of the DNA from its polymer complex if it is to be successfully transcribed.

However, some studies of PEI-DNA complexes have reported the complexes to be present in the nucleus in undissociated form, and thus unpackaging of the complexes must occur within the nucleus for these systems[112, 113]. Although the mechanisms remain unclear, DNA polymerase may be involved, and it has been postulated that once inside the nucleus, polyanions such as endogenous DNA replace the transfected DNA within the complex in a polyion-exchange reaction, releasing the therapeutic DNA within the nucleus. Synthetic polycations such as these may also behave in a way that mimics nuclear homing devices. Polycations may also be removed during transcription of nucleic acid by polymerases. Indeed, when injected directly into the nucleus, DNA complexed to PEI can be transcribed as efficiently as naked DNA. This was demonstrated by the work of Bieber *et al*[114] who showed that DNA tightly complexed with PEI (at high N:P ratios) was transcribed DNA as well as naked DNA, and more efficiently as more loosely associated complexes at a low N:P ratios. This group therefore proposed that DNA does not need to be dissociated from its polycation before it is transcribed[115, 116]. It can thus be concluded that nuclear localisation rather than DNA unpackaging in the nucleus is a key limiting factor in polymer-mediated gene delivery, and as a consequence, design criteria for new polymer vectors, especially those that invoke responsive or activated mechanisms, need to take into account the need for nuclear targeting. Nuclear transport can be enhanced by modification of synthetic polymers with nuclear localisation signal peptides[117-119], or with transcription factors, but of course this adds to the design and synthesis complexity of the vector system.

1.4. Applications of biodegradable polymers as gene delivery system

Biodegradable polymers provide potential advantages over non-degradable polymer for use in drug or gene delivery systems because of their good safety profile. Degradation of the polymer leads to non-toxic products which can be easily eliminated from the cell. Moreover, the degradation of the polymer can be used as a tool to control the release of the drug or biomolecules from the delivery system.

Polymer composed of lactide and glycolide such as poly (lactic acid), poly (glycolic acid) and the copolymers of lactic and glycolic acid, i.e., poly (lactide-co-glycolide). (PLGA) are most widely used in many biomedical applications (e.g. suture and implants or drug delivery (e.g peptide and proteins)[120]. PLGA polymers produce lactic acid and glycolic acid upon hydrolytic degradation, these two substances are naturally occuring in metabolic pathways of the body, therefore, their applications as drug and gene delivery systems are generally considered to be safe. PLGA polymers are commercially available from various vendors as good manufacturing practice (GMP)-grade.

The major supplier for PLGA of GMP grades are Purac (Trade name: Purasorb); Absorbable Polymers International, a wholly owned international subsidiary of Durect Corporation (Trade name: Lactel); Alkermes (Trade name: Medisorb); Boehringer Ingelheim (Trade Name: Resomer). PLGA polymers have been approved by US FDA for human use. PLGA polymers can also be synthesized by ring opening polymerization of lactide and glycolide. The degradation rate of the polymer can be controlled via the molecular weight of the polymer and also through lactide:glycolide ratio.

Usually low molecular weight and high content of glycolide accelerate the degradation rate. PLGA polymer composed of 50:50 ratio of lactide to glycolide is known to have a faster biodegradation rate in a bout 50-60 days, whereas other combinations of lactide:glycolide ratio of 75:25, or 80:20 are shown to have significantly longer degradation rate.

1.5. Micro- and nanoparticles based gene delivery system

Polymer based micro and nanoparticles provide several advantages for the delivery of macromolecules such as DNA. Polymeric delivery systems provide protection against nuclease degradation by encapsulation of the drug and macromolecules in the polymeric matrix[121]. Control over the release of drug or macromolecules (e.g. DNA) into a particular tissue or organs can be achieved via careful tailoring and choice of the polymers and thus controlling the rate of release from the delivery system.

The rate of release can be designed to occur rapidly for bolus delivery or can be adjusted to obtain release over a long period of time (sustained release). Sustained release of certain therapeutic molecules is extremely important particularly in the case of drugs with short half-lives or with narrow therapeutic windows in which case the release can be adjusted to maintain the concentration of the drug in circulation at desirable level.

Incorporation of plasmid DNA and oligonucleotides into a matrix of polymer micro and nanoparticles has been investigated widely. Various natural and synthetic polymers can be formulated as matrices and microspheres[122, 123].

There are a few parameters affecting the release of DNA from the polymer delivery systems, chemical composition of the polymer (esters) affects the degradation time and can be used to control the release rate. Secondly, physical properties such as size, mass and porosity can also affect the release of DNA[121]. The structures of some polymers that have been used in microparticle formation for DNA delivery are shown in Figure 1-2

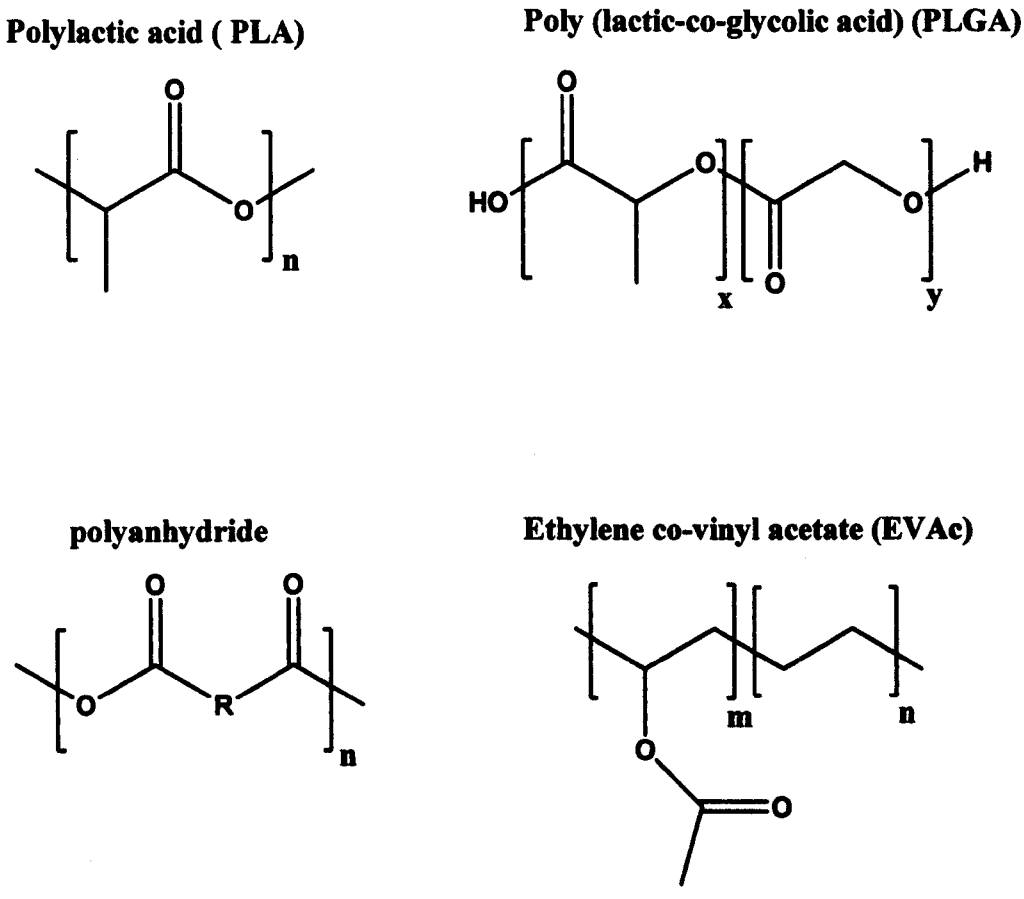


Figure 1-5. Structure of polymeric materials used for gene delivery.

PLGA-based polymers have been widely used in developing micro/nanoparticles for encapsulating therapeutic molecules for controlled release applications due to their inherent advantage over conventional devices including the extended release rates from days to months[124-127]. Macromolecules such as proteins, peptides, plasmid DNA, human growth hormone factor, etc, have been successfully incorporated into PLGA based micro/nanoparticles[128-130]. There are list of FDA approved controlled released products based on PLGA in the market (table 1-I).

Table 1-I. PLGA-based microparticles available in market.

Product name	Active ingredient	Company	applications
Lupron Depot®	Leuprolide acetate	TAP	Prostate cancer
Nutropin Depot®	Growth hormone	Genentech	Pediatric growth hormone deficiency
Suprecur® MP	Buserelin acetate	Aventis	Prostate cancer
Decapeptyl®	Triptorelin pamoate	Ferring	Prostate cancer
Sandostatin LAR® Depot	Octreotide acetate	Novartis	Acromegaly
Somatuline® LA	Lanreotide	Ipsen	Acromegaly
Trelstar™ Depot	Triptorelin pamoate	Pfizer	Acromegaly
Arestin®	Minocycline	Orapharma	Periodontal disease
Risperidal® Consta™	Risperidone	J & Johnson	Antipsychotic

Generally, micro - and nanoparticles are classified based on their sizes, microparticles have diameter in the range of 1 to 250 μm, whilst the size of nanoparticles are considered to range between 10 to 100 nm.

It has been reported that microparticles with diameters larger than 1 μm are internalised mainly through phagocytosis preferentially by antigen presenting cells (such as macrophages and dendritic cells), but not by other cells, therefore, biodegradable DNA microparticles have been especially investigated for (DNA) vaccination purposes against viruses and tumours as antigen presenting cells play a pivotal role in initiating of immune responses.

On the other hand, the submicron sized nanoparticles offer important advantages over microparticles in that their cellular uptakes are relatively higher. Studies showed that nanoparticles of 100 nm size have higher cellular uptake by 2.5 fold when compared to 1μm and 6 fold higher when compared to 10μm microparticles in Caco-2 cell lines[131]. Similar results were also obtained were tested in a rat *in situ* intestinal loop model. The efficiency of uptake of 100nm size particles was 15-250 fold greater than larger size (1 and 10 μm) microparticles[132].

1.5.1. Fabrication of DNA loaded PLGA nanoparticles, challenges and opportunities

There are a number of approaches described for encapsulating plasmid DNA within PLGA based nanoparticles, these include spray drying, phase separation methods and double emulsion techniques[133-136]. Initially, these approches have been used in encapsulation of peptide and proteins within the PLGA based micro

and nanoparticles. The double emulsion technique refers to a process where the peptide, proteins, or plasmid DNA are dispersed in the organic phase to make a water-in-oil emulsion using homogenisation or sonication. In the phase separation method, the particles are produced by extracting the organic solvent or adding non-solvent to induce coacervation, whilst in spray drying techniques, the particle formulation is achieved by atomising the emulsion through a stream of hot air to induce rapid solvent evaporation. In addition to the approaches mentioned above, plasmid DNA can also be coated on the surface of PLGA particles.

1.5.1.1. Double Emulsion Technique

Double emulsions are typically formed by mixing or dispersing of an aqueous solution of DNA with a solution of PLGA dissolved in an organic solvent. The dispersion of the DNA solution is usually performed by using sonication or homogenisation techniques.

This results in the formation of first or primary emulsion in which the aqueous droplets containing DNA are suspended in the organic phase. The size of the droplet formed by sonication or homogenisation can be optimised by adjusting the amount of the shear force used for dispersion process.

The primary emulsion is then mixed with a second aqueous solution that contains surfactants. Surfactants are usually used to prevent the aggregation of aqueous droplets (coalescing). The addition of primary emulsion with the solution of surfactant results in precipitation of polymer around the aqueous droplets and finally the evaporation of the organic solvents lead to the formation of solid

particles in which the DNA is embedded and trapped in the polymeric matrix of the particles.

PLGA based particles are sensitive to moisture and undergo hydrolysis upon contact with water molecules, therefore, it is important to keep the final particles free of moisture. Drying of the particles can usually be done by using lyophilisation or freeze drying for long-term storage. Sometimes, addition of cryoprotective sugars (usually sucrose or glucose) can be added to the formulation to preserve the DNA supercoiling during the drying process[137].

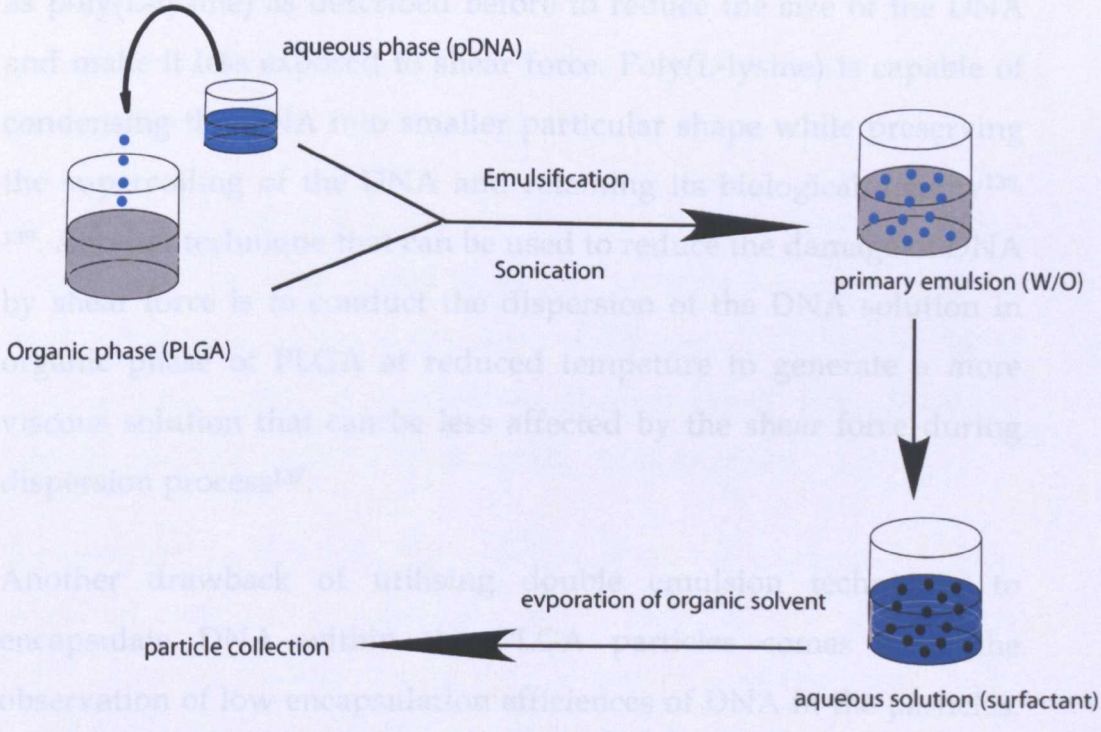


Figure 1-6. Encapsulation of DNA in PLGA nanoparticles using double emulsion technique.

One of the major drawbacks of double emulsion technique is usually due to the shear force used to disperse the aqueous solution of DNA in the organic phase. The shear force can damage the DNA and reduce the integrity and, eventually, the biological activity. In addition to that, contact of DNA with the organic phase also can damage the DNA therefore it is important to keep the amount of the shear force to minimum to retain the DNA supercoiling which represent the most stable form of DNA.

There are some methods that can be used to minimise the DNA damage by utilising shear force during dispersion process. Condensation of DNA can be carried out with cationic polymers such as poly(L-lysine) as described before to reduce the size of the DNA and make it less exposed to shear force. Poly(L-lysine) is capable of condensing the DNA into smaller particular shape while preserving the supercoiling of the DNA and retaining its biological activity[138, 139]. Another technique that can be used to reduce the damage of DNA by shear force is to conduct the dispersion of the DNA solution in organic phase of PLGA at reduced tempeture to generate a more viscous solution that can be less affected by the shear force during dispersion process[137].

Another drawback of utilising double emulsion techniques to encapsulate DNA within the PLGA particles comes from the observation of low encapsulation efficiences of DNA in the particles. This is partly due to, the leakage of DNA from the aqueous droplets during the formation of the primary emulsion and secondly, during the evaporation of the organic solvent.

1.5.1.2. Spray Drying Technique

In spray drying techniques, the DNA loaded particles are produced by combining emulsification steps with spraying via an atomizer through a steam of hot air. Initially, the DNA solution is mixed with PLGA solution in organic solvent using shear force to form droplets of DNA in the PLGA solution. Finally, the particles are formed by spraying the suspension of DNA droplets in PLGA solution through an atomizer in the presence of hot air. During spraying of the suspension through the hot air, the organic solvent and water moisture evaporate which results in the formation of dried solid particles loaded with DNA. In spray drying techniques, there are a number of factors contributing to the particles size of the final products such as the flow rate through the atomizer, temperature of the air and size of the DNA droplets[140].

In spray drying processes, the addition of primary emulsion into a second aqueous phase is absent, which could be an advantage over double emulsion techniques because of the loss of DNA in the latter technique. However, the mechanical, thermal stress effects upon the integrity of DNA could be disadvantage of the system.

Furthermore, in this way the plasmid DNA may indeed be better protected during the preparation of the particles, one should note that *in vivo* DNA associated to the surface may become more easily degraded and may result in a significant burst release.

1.5.1.3 Adsorbtion of DNA on surface of PLGA particles

In this technique particles are also made by double emulsion technique in the absence of DNA to produce empty PLGA particles, but in the second aqueous phase of double emulsion technique, the particles are suspended in solution of cationic agents which coat the outer surface of the particles. By incubation of these positive surface charges particles with solution of DNA, the DNA binds to the external surface through electrostatic interaction. In this type of procedure usually the loading efficiency of DNA is limited to 1% (weight/weight)[141].

1.5.2. Challenges associated with PLGA particles as nonviral gene delivery systems

Generally, nonviral gene delivery systems are significantly less efficient in comparison to viral gene delivery system. Therefore, for PLGA particle system to be considered as relatively efficient gene delivery systems they should fullfil some basic requirements. First of all, considering the low transfection efficiency of nonviral delivery systems, the particles should be able to carry a high amount of DNA which is termed as "loading efficiency". Secondly, as has been mentioned earlier, the integrity of DNA or the supercoiled structure of DNA should be preserved during the PLGA particle formulation i.e. all the detrimental conditions of high shear forces and thermal stress should be avoided or reduced to minimum to avoid any damage to DNA[142].

Considering the *in vivo* transfection efficiency, the PLGA particles should be able to protect the DNA from extracellular nuclease enzyme to avoid the rapid degradation before reaching the desired site of action. Finally, premature release of DNA from the particles, termed as "burst release", should be kept to as low as possible to maximise the amount of the payload at the site of action.

Another problem associated with PLGA nanoparticles is the hydrolysis of the PLGA backbone upon contact with water molecules. The hydrolysis process involves water molecules carrying out a nucleophilic attack on the ester bonds between the lactide and glycolide units in the backbone of PLGA polymer generating lactic and glycolic acid units[143]. This result in the degradation of the polymer and subsequently may lead to substantial reduction in pH and generation of acidic climate in the PLGA matrix where the DNA molecules are embedded. The formation of the acidic climate may cause degradation of DNA, compromising the biological integrity and eventually its therapeutic effect[136, 144].

It has been reported that the interaction between the PLGA based particles with the *in vivo* components can also compromise their efficiency as drug and gene delivery system. When these particles are administered intravenously, they can be easily recognised by the body's defence system. Mononuclear phagocyte system (MPS) identify PLGA based particles and rapidly clear them from blood[145]. The mechanism that involves the recognition of PLGA based particles by phagocyte can be attributed to the hydrophobic properties of the particles that allow the adsorption of blood components on their surface, such as opsonins, this is regarded as the first step toward elimination of the particles from blood.

It has also been reported that particles made of PLGA undergo significant aggregation and flocculation in biological media which are rich in enzymes (e.g gastrointestinal fluid)[146].

Finally, PLGA particles often have slow release profiles which could be a problem in some applications such as vaccinations in which the subsequent release of antigen from the particles is required to obtain an optimum immune response.

Therefore, there is a clear indication to develop a new type of biodegradable particle that can match all the basic requirements of an efficient carrier for gene delivery. Ideally, the new particulate gene delivery system should fullfil the following criteria:

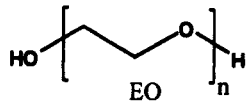
Requirements of efficient particulate gene delivery system

- The integrity of DNA preserved throughout the formulation process
- Efficient and high incorporation of DNA within the particles
- Degradation of the matrix of the particles should not generate any hostile (e.g. internal acidic environment) that can compromise the biological activity of DNA
- Absence or minimised premature release of DNA in circulation and enhanced release inside the cell
- Development of surface-modified nanoparticles to achieve longetivity in blood circulation
- Specific targeting, the particles loaded with DNA should be able to deliver the payload inside the desirable cell population.

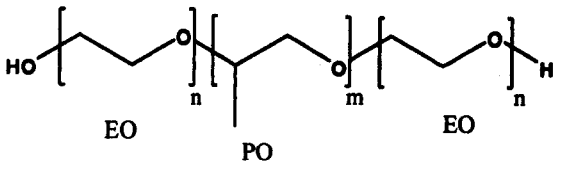
1.6. Application of polymers for designing advanced nanoparticulate gene delivery system

1.6.1. Poly(ethylene oxide and derivatives

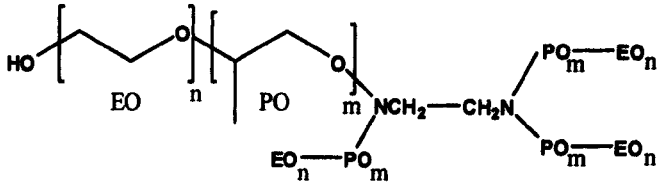
The term poly(ethyle oxide) (PEO) and poly(ethylene glycol) (PEG) refer to the same material, although the term PEO is usually preferred when describing high molecular weight polymer. The derivatives of PEO which are composed of poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) units are named commercially as poloxamers and poloxamines. The difference between the two molecules are mainly in their chain length and PEO, PPO contents. Furthermore, Poloxamines differ from poloxamers in that a molecule of ethylendiamine links the components together. The structures of these two polymers are shown in figure.



Poly(ethylene glycol) or Poly(ethlylene oxide)



Poloxamer



Poloxamine

Figure 1-7. Chemical structure of PEG and some commercial derivatives Poloxamers, Pluronics and Poloxamines.

The potential of PEO and its derivates (poloxamers and poloxamines) as biomaterials has been widely investigated. Poloxamers and poloxamines have relatively good biocompatiblity and low toxicity which make them very attractive biopolymers to use in many applications such as drug and gene delivery system. However, these polymers are not biodegradable which make their application for drug delivery dependent on the molecular weight.

High molecular weight PEO of about 7000 kDa is known to be acceptable for application of oral drug delivery system[147, 148], whilst the lower molecular weights (preferably 5 - 10 kDa) have been suggested for use in parenteral adminstration[149, 150].

The potential of poloxamers, in this particular case, have also been investigated for use as gene delivery systems. It has been shown that poloxamer polymers (Pluronic® L61 and Pluronic® F127) have the ability to complex with DNA molecules and to protect the plasmid DNA from nuclease enzyme which resulted in enhancement of gene expression[151, 152].

Another potential application of PEO and its derivatives (poloxamers and poloxamines) is for surface modification of drug and gene delivery systems such as micelles, liposomes and nanoparticles. As has been mentioned earlier, uncoated drug delivery systems such as PLGA particles suffer a rapid blood clearance after their intravenous administration owing to the fact that opsonins adsorb on the surface through hydrophobic interactions and consequently they are taken up by mononuclear phagocyte system (MPS) and finally eliminated from circulation.

Therefore, PEO and its derivatives have been of interest for use in surface modifications of drug delivery systems, this is due to their neutral charges, hydrophilicity and flexible chains which can form a " steric shield" on particle surfaces.

1.6.1.1. Surface modification of PLGA based particles with PEO and its derivatives

Incorporation of PEO and its derivatives into PLGA based particles offer potential advantages to improve their efficiency as drug and gene delivery systems. Originally, PEO and its derivatives were incorporated into PLGA particles for two main reasons. Firstly, preventing the hydrophobic PLGA particles from interaction with each other causing aggregation, this is prevented by providing the particles with a protective hydrophilic stealth. As a result of that, opsonisation and rapid clearance of PLGA particles from the blood circulation can also be avoided.

Secondly, coating of PLGA particles with functional PEO can facilitate the introduction of ligand (targeting moiety) on the surface of particles to produce an active-targeting delivery system[153, 154]. In addition to that, by blending of PEO and its derivatives with PLGA based particles, it is believed that it is possible to improve the stability of encapsulated molecules. In addition, co-incorporation of other PEG based components, such as cationic Jeffamines or poloxamines, provides a more friendly environment for DNA in the matrix of the particles by buffering the acidification that occurs during degradation of PLGA polymer, more importantly, they can enhance the encapsulation efficiency and release profile.

There are two main approaches to incorporate PEO and its derivatives, poloxamers and poloxamines, into PLGA based particles. The first approach includes physical entrappment or adsorption of PEO and its derivatives into or onto PLGA particles. The second approach includes chemical modifications of PLGA polymer via covalent linking with PEO. Illustration is shown in Figure 1-8.

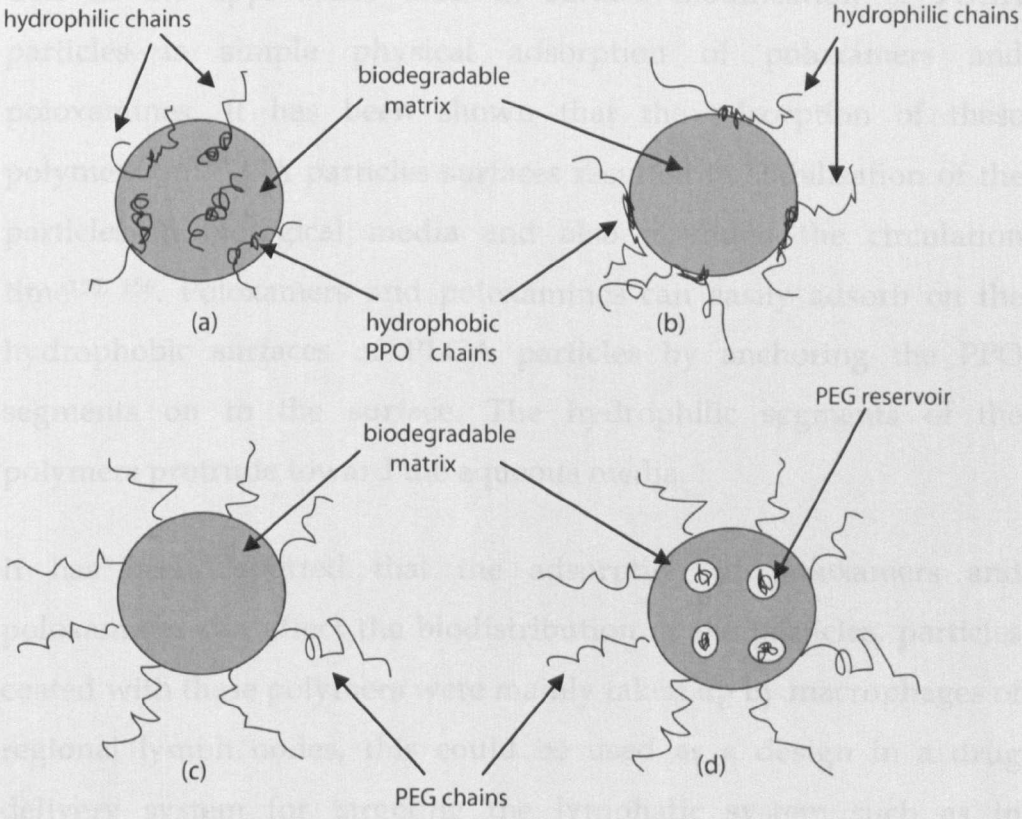


Figure 1-8. Schematic view of various structure of nanoparticles made of PLGA with PEO and its derivatives (poloxamers and poloxamines), (continued next page).

(a) Poloxamers/poloxamines blended with PLGA (b) poloxamers/poloxamines physically adsorbed on PLGA nanoparticles (c) and (d) Nanoparticles made of PEG-PLGA copolymers.

1.6.1.2. Adsorption of PEO and its derivatives on surface of PLGA particles

One of the approaches used in surface modification of PLGA particles is simple physical adsorption of poloxamers and poloxamines. It has been shown that the adsorption of these polymers on PLGA particles surfaces resulted in stabilisation of the particles in biological media and also extended the circulation time[155, 156]. Poloxamers and poloxamines can easily adsorb on the hydrophobic surfaces of PLGA particles by anchoring the PPO segments on to the surface. The hydrophilic segments of the polymers protrude toward the aqueous media.

It has been reported that the adsorption of poloxamers and poloxamines can affect the biodistribution of the particles, particles coated with these polymers were mainly taken up by macrophages of regional lymph nodes, this could be used as a design in a drug delivery system for targeting the lymphatic system such as in vaccination[157, 158].

One of the main drawbacks of this approach comes from the displacement of poloxamers and poloxamines polymers from the surface of PLGA particles by serum proteins[159]. This effect on stability of the particles, prompts the particles toward aggregation and, finally, opsonisation and elimination from circulation.

The alternative approach would be to coat the particles with PEO-based polymers via chemical modifications. In this way it is possible to covalently link the PEO polymers to PLGA polymers that form the matrix of the particles or via synthesis of copolymers of PLGA with PEO using polymerisation techniques.

1.6.1.3. Blend of PEO and its derivatives within the matrix of PLGA particles

In this approach poloxamers and poloxamines are physically mixed with PLGA polymer to form particles of blended-type matrices. This type of particles are usually prepared by using double emulsion techniques to encapsulate hydrophilic molecules within the matrix. It has been shown that particles of PLGA and poloxamers blend matrix are able to stabilise the encapsulated molecules and improve the release profile[160, 161].

Two types of blend matrices have been mentioned. First, the particles are prepared by double emulsion-solvent extraction/evaporation technique. In this example the poloxamers were added to the internal aqueous phase and during precipitation of PLGA polymers, the poloxamers were entrapped in the form of small reservoirs. This type of preparation is important particularly when the encapsulating molecules are co-dissolved with the poloxamers in the internal aqueous phase[162].

More recently, double emulsion solvent diffusion techniques have been investigated to prepare particles with a blend matrix of poloxamers and PLGA polymers. In this particular case, both PLGA and poloxamers/poloxamines are co-dissolved in the organic phase and the co-precipiration upon addition to miscible organic solvent.

The formation of this type of blend matrix is based on the hydrogen bonding between the poloxamers / poloxamines and PLGA[163, 164] Figure 1-9.

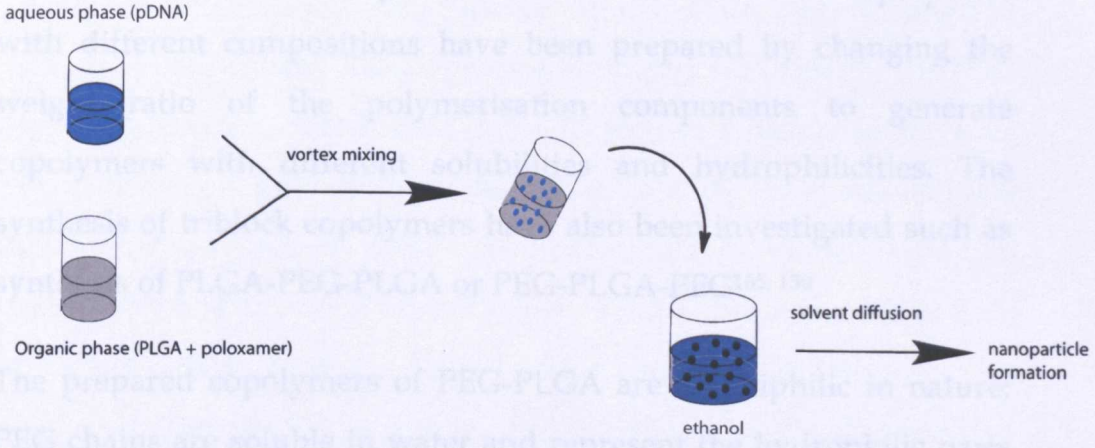


Figure 1-9. Encapsulation of pDNA into PLGA/poloxamer blend nanoparticles by modified emulsification-solvent diffusion.

1.6.2. Chemical modification of PLGA polymer via covalent linking with PEO

Surface modifications of PLGA particles with poloxamers and poloxamines using physical adsorption technique have been shown to be an inefficient approach to provide stability to the particles, due to displacement of the polymers from the surface of the PLGA particles by serum components[159]. Therefore, an alternative approach to surface-modify the PLGA particles with stable PEG chains includes covalent attachment of PEG to PLGA polymers.

One of the approaches to perform this covalent attachments of PEG to PLGA is via ring opening polymerisation technique (ROP).

Monomethoxy-poly(ethylene glycol) is used as initiator to polymerise the monomers of lactide and glycolide in the presence of stannous octoate as catalyst[149]. A series of PEG-PLGA copolymers with different compositions have been prepared by changing the weight ratio of the polymerisation components to generate copolymers with different solubilities and hydrophilicities. The synthesis of triblock copolymers have also been investigated such as synthesis of PLGA-PEG-PLGA or PEG-PLGA-PEG[165, 150].

The prepared copolymers of PEG-PLGA are amphiphilic in nature; PEG chains are soluble in water and represent the hydrophilic parts whereas PLGA parts are hydrophobic and insoluble in water. Copolymers of PEG-PLGA have been used to prepare nanoparticles using emulsion techniques in exactly analogous ways as described before such as solvent evaporation of solvent diffusion techniques.[166, 167] Because of differences in solubilities of PEG and PLGA polymers, a phase separated structure can be formed at the interphase of organic phase and aqueous phase during particle preparation process. The PEG chains project toward the aqueous medium while covering the PLGA solid core forming "core-shell" particles[149].

It has been reported that the organisation of PEG-PLGA copolymer within the matrix of the particles depends on the preparation techniques. Using double emulsion-solvent evaporation techniques, part of the PEG chains are likely to be physically oriented toward the internal aqueous phase during the formation of primary emulsion. Thus, upon precipitation of PLGA polymer during the addition to external phases, small PEG reservoirs are formed.

The PEG reservoirs in the matrix of PLGA particles can offer numerous advantages, PEG reservoirs can increase the stability of encapsulated hydrophilic molecules by providing a more compatible environment such as in the case of encapsulation of DNA or proteins. They may increase the encapsulation efficiency of the hydrophilic molecules by reducing the leakage of the molecules to external aqueous phase during PLGA precipitation process. Finally, the PEG reservoirs are associated with water molecules which may have an effect on the degradation of the solid core of the particles and influencing the release profile of hydrophilic molecules from the particles[168]. Example of these effects have been confirmed in the case of encapsulation of tetanus toxoid which retained biological activity when incorporated in the matrix of PLGA-PEG-PLGA microparticles and PLA- PEG nanoparticles[169].

1.7. Synthesis of amphiphilic block co-polymers

Recently, considerable interest has been paid to synthesis of amphiphilic block copolymers. The difference in solubility of the hydrophobic to hydrophilic block leads to self-assembly in aqueous media to form polymeric micelles with controllable sizes.

The biomedical applications of these block copolymer micelles have rapidly increased as it has been realised that a novel carrier systems for the targeting of poorly soluble drugs might be realised block co-polymers have a unique ability to encapsulate large of amount of insoluble drugs in the hydrophobic core and the ability to tune hydrophobic and hydrophilic balance enables control over drug release and biodistribution [170, 171].

The distribution of these polymeric micelles in the body may be dependent upon their size and surface charge. In this regard, the ability to control the size and surface charges of these nanocarriers also has crucial effect on *in vivo* therapeutic efficacy[170]. Since the discovery of living free radical polymerisation methods, block copolymers have become easily accessible with control over the molecular weight of the block copolymer and the ratio between the blocks. Block co-polymers can also be obtained with narrow molecular weight distribution. The main types and most widely used living free radical polymerisation techniques are atom transfer radical polymerisation (ATRP) and reversible addition fragmentation transfer (RAFT)[172, 173].

As has been mentioned earlier, materials based on poly(ethylene glycol) (PEG), poly(lactic acid) (PLA), (PGA) and their co-polymers have been widely explored for biomedical use, particularly for drug delivery[174, 175]. The 'stealth' properties of PEG, which have been utilised to enhance pharmaceutical profiles of therapeutics ranging from small molecules to proteins[5, 176, 177], can be combined with the controllable biodegradability of PLA/PGA co-polymers (PLGA) to yield highly effective carriers for a variety of drug compounds[178].

PLGA–PEG block co-polymers can form micelles in aqueous solutions or can be produced as nanoparticles with solid cores, enabling them to encapsulate drugs with widely differing solubility properties[179, 180]. However, fine-tuning of the properties of these co-polymers is more difficult owing to the limited availabilities of PEGs with appropriate functional chain ends and ranges of molar mass.

By contrast, PEG side-chain polymers, exemplified by PEG-methacrylate (PEGMA), can be synthesised via a variety of routes, and very fine manipulation of properties can be exerted through controlled polymerisation strategies.

Controlled radical polymerisations, primarily ATRP [181, 182] and RAFT[183, 184], have enabled PEG-methacrylates and co-polymers with finely controlled properties to be produced[185, 186]. One of the aims of this work was to show that a combination of ring-opening polymerisation with RAFT agent initiated growth of PEG-methacrylate can yield co-polymer micelles with controllable properties, and with promise as drug-encapsulation and release systems.

1.7.1. Ring Opening Polymerisation technique

As was mentioned above, the biodegradable polymers poly(D,L-lactide-co-glycolide) (PLGA) have considerable importance in several medical applications including the controlled release of drugs[187], absorbable sutures, and tissue engineering[188]. The advantages of PLGA for these applications stem from the polymer's biocompatibility, degradation, processability, mechanical strength, and its approved use by the FDA for *in vivo* use[189].

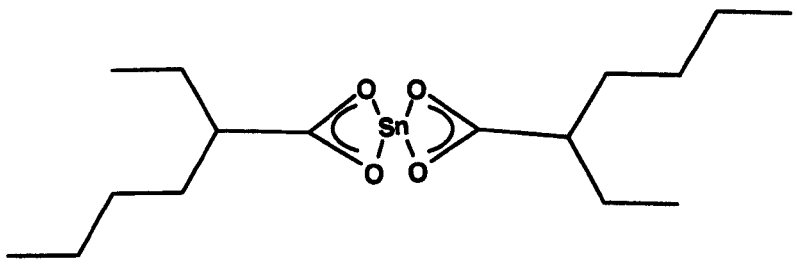
PLGA polymer can be synthesised via one of two mechanisms, either condensation reaction with lactide and glycolide monomers, ring opening polymerisation of lactide and glycolide. The polycondensation method is known to be an equilibrium, and it is difficult to remove the liberated water and this limits the molecular weight of the polymer.

However, this problem can be circumvented by an azeotropic distillation process as patented by Mitsui Toatsu Chemicals Inc[190, 191].

The ring-opening polymerisation of lactide and glycolide allows for a much higher control of the polymerisation than polycondensation. As a result, ROP is the most widely used method for the synthesis of well-defined materials. In practice, a suitable polymerisation conditions allow for the controlled ring opening polymerisation of lactide and glycolide, and the mean degree of polymerisation (DP) of the resulting polymers is usually equal, or at least proportional, to the monomer conversion times the monomer to initiator molar ratio.

Polymerisation can occur either in bulk or solution; however, bulk polymerisation is desired when the end product is intended for medical applications. Bulk polymerisation eliminates the use and potential residual presence of organic solvents such as toluene, benzene, or chlorobenzene in the polymer[192]. In addition to that, compared to condensation reactions, ROP is much more convenient for the control of monomer sequences, for example during block copolymer synthesis as well as of the polymer chain ends[193]. ROP of lactide and glycolide requires a suitable catalyst to open the rings and initiate the polymerisation in a controllable manner.

The most widely used catalyst for industrial preparation of lactide and glycolide polymers is tin (II) bis (2-ethylhexanoate), (Figure 1-10). Usually referred to as tin (II) octanoate, Sn (Oct) $_2$, is commercially available, easy to handle, and soluble in common organic solvents and in melt monomers. It is highly active (typical reaction times in bulk at 140-180 °C range from minutes to a few hours) and allows for the preparation of high-molecular-weight polymers (up to 10^5 or even 10^6 Da in the presence of an alcohol)[194]. Sn (Oct) $_2$ has been accepted as a food additive by the U.S. FDA.



$Sn(Oct)_2$

Figure 1-10. Structure of tin (II) octanoate [Sn (Oct) 2.

Other catalysts mentioned to be efficient in ROP are aluminium (III) isopropoxide [Al (Oi-Pr) 3], and zinc (II) lactate [Zn (Lact) 2]. Sn (Oct) 2 is known to be more active than Al (Oi-Pr) 3 which usually needs a longer reaction time. $Zn(Lact)_2$ is relatively good polymerisation catalyst and its known to be more active as $Al(Oi\text{-}Pr)_3$[195, 196].

It has been reported that ROP polymerisation is faster and better controlled when $Sn(Oct)_2$ was combined with a protic reagent such as alcohol[193].

The coordination-insertion mechanism for the ROP of cyclic esters was first formulated in 1971 by Dittrich and Schulz[197]. Support for this mechanism has been obtained theoretically for $Sn(Oct)_2$-catalyzed ROP of Lactide in the presence of methanol[198].

The mechanism of reaction is described as two molecules of methanol coordinated to Sn (Oct) 2. Both coordinations occur in an associative fashion, i.e., with retention of the two octanoate moieties (hydrogen bonds are formed between the alcohol and octanoate ligand). A weak complexation of lactide occurs; the latter coordination step induces a proton migration from methanol to the nearby octanoate ligand, so that the alcohol ligand is converted into an alkoxide. Subsequently, the insertion occurs in two steps, namely,

nucleophilic attack of this alkoxide on the coordinated lactide followed by ring opening

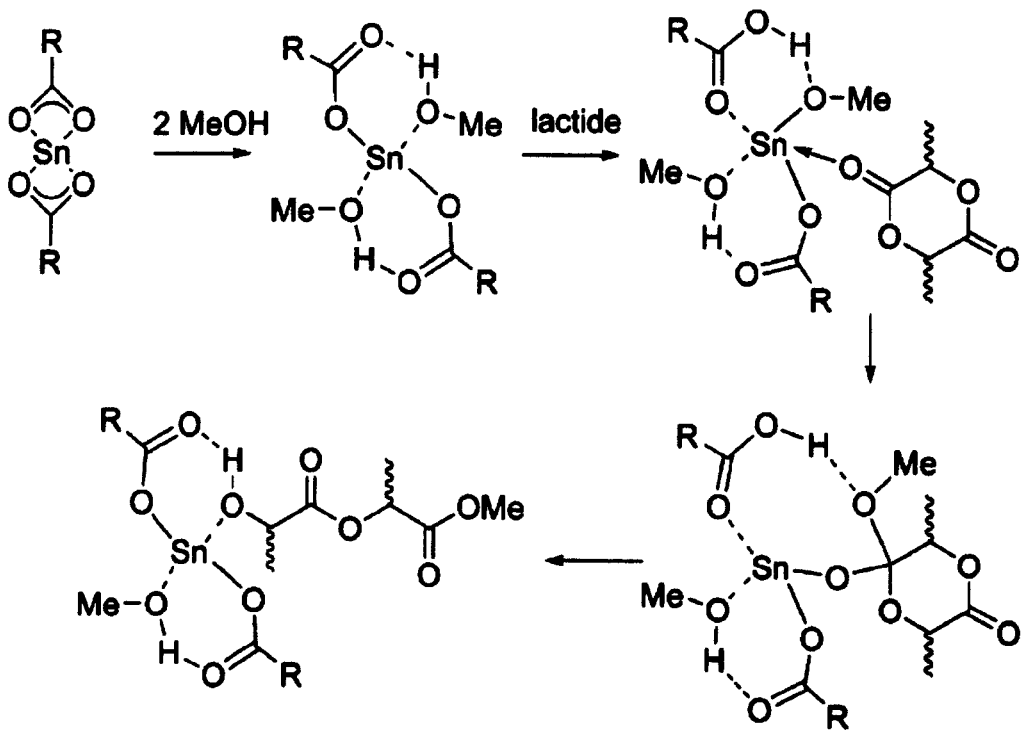


Figure 1-11. Predicted Mechanism for the Sn $(Oct)_2$-catalyzed ROP of lactide in the presence of methanol (R= Me)[193].

1.7.2. Living radical polymerisation

1.7.2.1. Atom transfer radical polymerisation (ATRP)

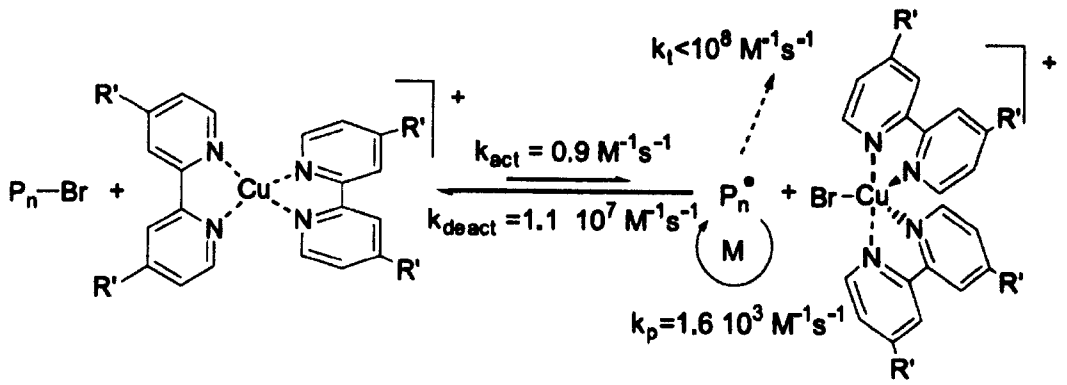


Figure 1-12. Principle of ATRP technique[199].

ATRP is a multicomponent reaction which consists of a transition metal species (Mt^n) capable of expanding its coordination sphere and increasing its oxidation number, a complexing ligand (*L*), and a counterion to form covalent or ionic bond with the metal centre. Other factors also important are solvent and temperature.

The alkyl halogen bond RX homolytic cleavage occur by the transition metal complex (Mtn /L) and this generates the corresponding higher oxidation state metal halide complex (Mt^{n+1X}/L) (this process occur with the rate constant of activation K_{act}) and an organic radical *R*•.Polymer chains grow by addition of organic radical *R*• to monomers in a manner which is similar to conventional radical polymerisation[200, 201]with a rate constant of (*Kp*).

Termination occurs as in free radical polymerisation via coupling or disproportionation (K_t) or reversible deactivation (k_{deact}) by (Mt^{n+1X}/L) which forms a halide-capped dormant polymer chain.

The rate of polymerisation can be obtained from the magnititude of the equilibrium constant (K_{eq}) which can be derived from an activation constant (K_{act}) and the deactivation constant (K_{deact}) as described in the equation below:

Equation of equilibrium constant in ATRP system

$$K_{eq} = K_{act} / K_{deact}$$

When the equilibrium constant K_{eq} is too small, in this case, ATRP will not occur or will occur very slowly. Whereas if the equilibrium constant is too large, this will result in a large amount of termination due to a high radical concentration. Consequently, a large amount of deactivating higher state oxidation metal complex will drive the equilibrium toward dormant species which result in slower polymerisation, but better control.

The atom transfer step is the key elementary reaction and is responsible for the uniform growth of the polymeric chain. In a well controlled ATRP, no more than a few percent of the polymer chains undergo termination. Typically, no more than 5% of the total growing polymer chains terminate during the initial stage of the polymerisation. As has been mentioned earlier, ATRP is composed of several components including monomers. It has been mentioned that a variety of monomers have been successfully polymerized by ATRP technique. Examples of those monomers are mainly vinyl based

monomers such as styrenes, (meth)acrylates, (meth)acrylamides, and acrylonitrile.

These monomers contain substituents to stabilise the propagating radical, and each monomer has their own radical propagation rate, therefore, it is important for a specific monomer, to adjust the concentration of propagating radicals and the rate of radical deactivation to maintain control over the polymerisation.

Using ATRP techniques, it is possible to pre-determine to a reasonable degree the M.W. of the final polymer via the degree of polymerisation (DP) which is set by the initiator concentration. Also, in a typical ATRP the molecular weight increases linearly with conversion and it is therefore possible to terminate the polymerisation at any point of time in order to achieve the desired molecular weight.

1.7.2.2. Radical addition-fragmentation termination polymerisation (RAFT)

After the intial report by the CSIRO group in 1998 the reversible addition fragmentation chain transfer (RAFT) has been recognised as one of the most versatile methods for imparting living characteristics to radical polymerisation and also providing control over the molecular weight and dispersity.

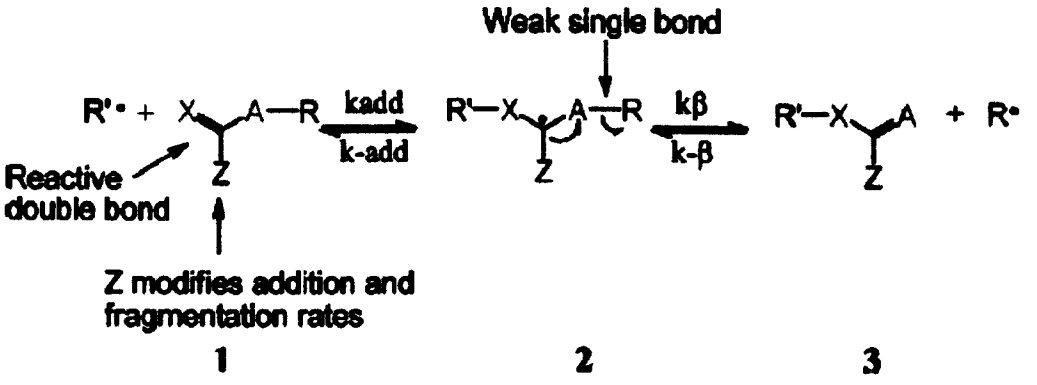


Figure 1-13. mechanism of addition fragmentation chain[202].

The RAFT agents are unsaturated compounds with general structure (1) shown in the (Figure 1-13). They act as transfer agent by a two-step addition-fragmentation mechanism.

In compound (1), the double bond (C=X) is reactive toward radical additon. The reactivity of the raft agent toward the propagating radical and the stability of the intermediate radical (2) is depends on a substituent Z. Whereas both A and X groups often referred to CH_2 or S. The group R is regarded a homolytic leaving group to generate *R•* which is capable of efficiently re-intiation of the polymerisation. Therefore, both compound (1) and compound (3) are considered as active transfer agents under the polymerisation condition[203, 202].

The general mechansim of RAFT polymerisation is shown as below

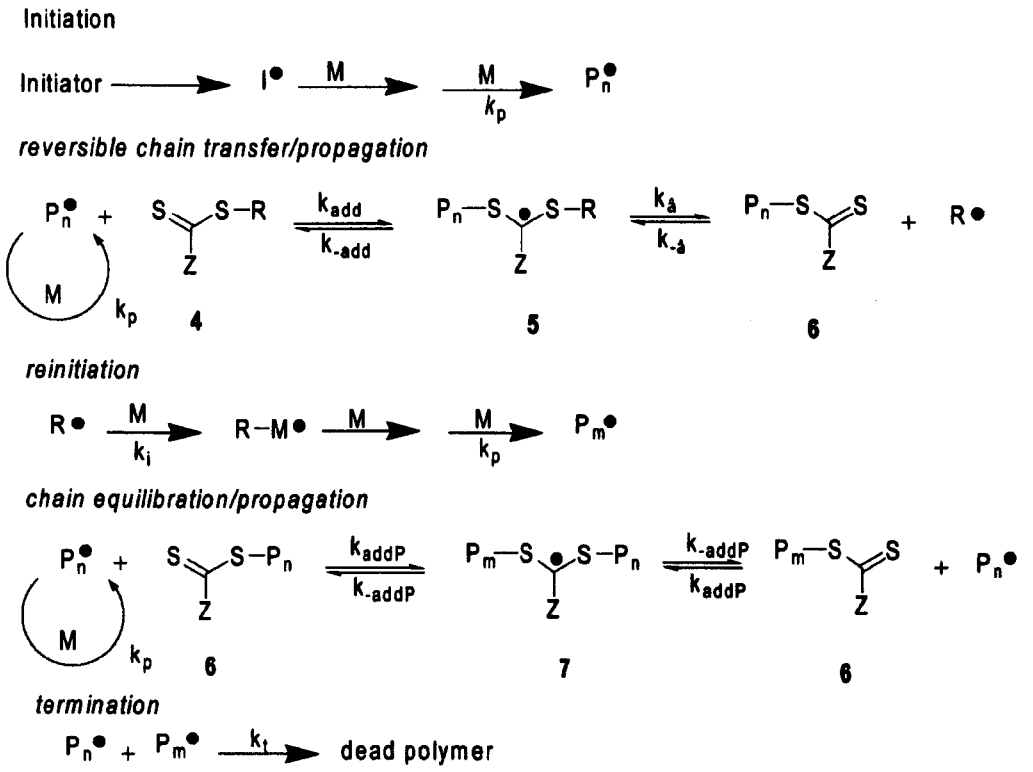


Figure 1-14. The general mechanism of RAFT polymerisation[202].

The general mechanism for RAFT process involves a sequence of addition-fragmentation equilibria as shown in (Figure 1-14). Similar to conventional radical polymerisation, the radical is generated by the initiator.

The generated radical is added to the transfer agent to form an intermediate compound which is subsequently followed by homolytic cleavage of the R group to form a new radical R•. Re-initiation by R• produce a new propagating radical P_m•.

Due to the rapid equilibrium between the active propagating radicals (P_n• and P_m•) and the corresponding dormant species 6 provides equal probability for all chains to grow and allows for the production of narrow polydispersity polymers. When the polymerisation is completed or (stopped), the majority of the polymer chain will retain the RAFT agent (thiocarbonylthio end group). The key element of RAFT polymerisation is that the thiocarbonylthio groups are retained in the polymeric product and this is characterstic of the living nature of the RAFT polymerisation. The RAFT agent at the end of the polymer chains also allows for synthesis of block copolymer synthesis even in one-step synthesis (assuming that all initial monomers are consumed before addition of the second).

As was mentioned earlier, the efficiency of the RAFT agents relies on both substituents R and Z groups. Almost all types of monomers can be polymerised by the RAFT process but a suitable RAFT agent is required to polymerise a specific monomer.

Generally, dithiobenzoates and other aromatic dithioesters, aromatic dithiocarbamates, trithiocarbonates are regarded as very efficient RAFT agents whereas for R groups, cyanoalkyl or cumyl moieties are the most common particularly for methacrylate based monomers due to their good captodative character[204].

1.8. Aim of the thesis

The aim of this thesis was to develop advanced nanocarriers based on synthetic biodegradable and biocompatible polymers with tailored physicochemical properties to fulfill the requirements of an ideal drug and gene delivery system.

In chapter two, the initial goal was the development of new a nanoparticulate carrier system for gene delivery purpose. The primary objectives were first, to formulate a blend matrix nanocarrier based on PLGA and polyethylene glycol derivatives (Jeffamine™). Secondly, to study the impact of the new blend system on the encapsulation efficiency of DNA, as well as the release profile. Finally, to coat the surface of particles with hydrophilic polymers to provide protective shells to enhace particles stability, as well as to increase compatiblity with blood components.

In chapter three, by exploiting modern polymerisation techniques (controlled living polymerisation techniques), the aim was to synthesise amphiphilic block copolymers based on PLGA and PEG-methacrylates to fine-tune properties and to use these copolymers in formulation of drug and gene delivery systems. It has been hypothesised that the new synthetic route would overcome the difficulties associated with the traditional methods to prepare PEG-PLGA copolymers owing to the limited availabilities of PEGs with appropriate functional chain-ends and ranges of molar mass. It has also been considered that by simply changing the length of the blocks and polymer compositions, the size, encapsulation efficiency and releasing profile of the nanocarrier systems can be easily tuned.

In chapter four, the ultimate aim was to develope multifunctional, bioresponsive and cell targeted nanocarriers, in a single construct, based on biodegradable and biocompatible PLGA and PEG-methacrylate copolymers that can be potentially used for drug and gene delivery systems.

The main objective of this work includes:

1- The synthesis of block copolymers based on PLGA and PEG-methacrylates using a combination of ROP and ATRP.
2- Synthesis of PLGA-S-S-PLGA block copolymers.
3- Incorporation of functionalities such as disulfide bridge between the blocks in the copolymer.
4- Functionalisation of block copolymers with a targeting moiety.
5- Combining the two copolymers using a double emulsion technique to formulate functionalised core-shell nanoparticles with DNA encapsulated in the matrix.
6- Examine encapsulation efficiency of pDNA.
7- Examine release profile in redox environment.
8- Study cellular uptake and viability on a clinically relevant cell lines.
9- Examination of incorporated fucntionalites on *in vitro* transfection efficiency.

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Chapter 2

Nanoparticulate gene delivery system

2. Introduction

The success of gene therapy is largely depends on the availability of an efficient gene delivery system, which will be able to protect and deliver the DNA into targeted tissue and cells[1-4]. From this point of view, viral vectors are considered as the "gold standard" and able to achieve the highest transfection efficiency[5]. This is related to their natural ability to cross extracellular and intracellular barriers effectively. However, viral vectors have several drawbacks in their application as gene delivery system, such as the induction of an immune response against the viral proteins, possible recombination with wild-type viruses, limitations in the size of inserted DNA, and difficult large-scale pharmaceutical grade production[6-9].

On the other hand, non-viral gene delivery displays potential advantages over viral gene delivery systems owing to their reduced toxicity, ease of scale-up, storage stability and improved quality control[10-12]. It should be noted, however, that the efficiency of non-viral gene delivery is still far below that of the viral ones.
One of the strategies aimed at the construction of a successful non-viral gene delivery has been the designing and preparation of polymeric micro- and nanoparticles[13].

Poly(DL-lactide)(PLA) and poly(lactic-co-glycolic acid)(PLGA) polymers have been widely used to prepare polymeric particles owing to their approved track record by the FDA for use as vehicles for drug and protein delivery[14-16]. Moreover, there are several ways to load the PLGA based particles with the therapeutic agents, physical entrapment, adsorption or chemical conjugation to PLGA matrix[15]. PLGA-based particles have also been shown to be able to escape from the endosomal compartments through surface charge reversion and to release the payload in cytoplasm in controlled manner[17-20].

PLGA-based particles are able to encapsulate plasmid DNA, protect it from nuclease degradation and increase its stability[21-23]. In addition, PLGA particles sized in the range of less than 10 μm can be efficiently phagocytosed by antigen presenting cells (APCs), therefore, their application for genetic vaccination has been widely explored[24, 25]. An example of this is the intramuscular immunisation of p55 Gag plasmid adsorbed on PLGA/ceytltrimethylammoniumbromide (CTAB) particles, which results in induction of potent immune response[26].

The mechanism of cellular uptake of PLGA particles is size dependent[27]; therefore, special interest has been paid to nanoparticles which are able to cross the biological barriers more effectively, hence improving the transport of encapsulated molecules. It has been mentioned that the cellular uptake of nanoparticles is non-specific and occurs either through clathrin vesicles, or by fluid-phase pinocytosis[17]. Upon internalisation of the nanoparticles, they are transferred to endosomal compartment where destabilisation of the membrane occurs owing to surface charge reversion on PLGA particles. After escape from the endosomal compartments, the PLGA particles release the encapsulated molecules into the cytosol in a controlled manner[18].

Despite the advantages of PLGA particles for use in gene delivery systems, particularly nanoparticles, there are significant barriers in the development of PLGA delivery systems. One of the main issues in a PLGA delivery system is the difficulty experienced with encapsulation of hydrophilic molecules like DNA within the hydrophobic matrices of PLGA particles. In an attempt to improve the encapsulation of DNA within PLGA nanoparticles, several methods have been introduced such as spray drying and oil in water solvent evaporation techniques[22, 28, 29]. However, during the encapsulation process of DNA using these methods, the DNA is liable to be damaged, and, also, degradation of PLGA polymer will result in the formation of an acid climate by the accumulation of the oligomers within the particles[22, 28]. Because of the hurdles and problems mentioned above, other approaches have been explored to improve the efficiency of PLGA to encapsulate and release the DNA whilst keeping the biological actively high.

There are several methods used in preparation of PLGA particles which including:

- spray-drying
- emulsion/evaporation
- double emulsion/evaporation
- salting out
- solvent displacement/nanoprecipitation
- emulsion-diffusion-evaporation techniques

The choice of these methods to prepare PLGA particles is mainly dependent upon the targeted size (particular size required) and the loading efficiency. For instance, in the case of genetic immunisation application, the particle size is required to be in the range of 1 - 10 μm

because the particles within this size range are shown to be taken up very efficiently by antigen presenting cells (APCs)[30].

Different particle sizes are required if the targeting cells are nonphagocytic cells, particles need to be in a size range of less than 300 nm, and uptake of these particles size is mediated by clathrin-endocytosis[31], (see section 1.3. in chapter one).

One of the commonly used methods to encapsulate DNA in the matrix of PLGA particles is the double-emulsion-solvent evaporation technique. This method consists of three main phases, an internal aqueous phase which usually contain a known amount of DNA to be loaded, an intermediate organic phase in which the PLGA polymer is dissolved and, finally, an external aqueous phase containing a known concentration of surfactant that will be used to stabilise the particles and prevent their aggregation[22, 32, 33].

In the process of PLGA particle preparation using double-emulsion-solvent evaporation technique, there are several parameters that affect on the particle size. Among these parameters, stirring rate and surfactant concentrations have been mentioned. PLGA particles have been prepared by this method and poly vinyl alcohol (PVA) chosen as surfactant in the external phase. By varying the concentration of PVA from 9% to 0.1% (w/w), the size of the particles has been controlled 1.6 - 9.7 μm, during which the stirring rate was fixed at 3400 round per minutes(rpm). However, the size of the particles decreased by increasing the stirring rate from 3400 to 1000 rpm whilst keeping the concentration of PVA surfactant at 0.1% (w/w)[34, 35]. Therefore, the size of the particles can be optimised by changing the parameters of a given method.

The approach of using double-emulsion-solvent evaporation technique to encapsulate DNA in the matrix of PLGA particles has many

concerns, particularly, the loading efficiency and preservation of biological activity of DNA during formulation process.

In the double emulsion technique, in order to obtain smaller size particles, usually a high shear force has to be utilised such as homogenisation or sonication. Theses high shear forces have a detrimental effect on DNA molecules and may compromise the biological activity. It has been reported that homogenisation and sonication used during emulsion techniques can convert supercoiled DNA into linear or circular conformation. Supercoiled DNA is known to be the most stable form and can produce a higher level of transfection compared with linear or open form DNA[36, 37]. Despite that, there are some reports indicating that after extraction, plasmids with a low supercoiled fraction have a biological activity which is equal or slightly less to DNA in supercoiled formation[27, 38, 39].
In contrast, the usage of sonication during emulsification technique has a much higher detrimental effect on the integrity of encapsulated DNA. Sonication can convert the double stranded DNA into a single stranded version[28]. The mechanism by which this damaging effect occurs is related to the formation of gaseous bubbles during sonication; (dispersion by sonication is based on shear force which originated by cavitations which causes the solvent as well as the molecules to become intensely agitated), upon collapse of these bubbles the medium becomes intensively agitated and results in the breakdown of the double stranded DNA. This is in addition to production of H_2O_2 in the process of water sonolysis[40], which could also be very harmful[41].

There are other modified methods derived from double emulsion techniques that can be utilised in the preparation of PLGA particles. These methods use the least amount of shear force to obtain particles

within nanoscale ranges. The spontaneous emulsification solvent diffusion method has been mentioned as one of the methods that require minimum shear force to produce nanoparticles[42-44]. The principle of this method was described in chapter one as an organic phase which consists of two organic solvents, one which has poor water miscibility properties (e.g. dichloromethane) and the other of which has good water miscibility (e.g. acetone). Upon the addition of these organic solvents into an aqueous medium (non solvent to PLGA polymer), acetone rapidly diffuses out into the aqueous medium, precipitating the dissolved PLGA polymer in the organic phases at the interfacial face and this diffusion results in the formation of particles in a nanoscale range.

As described above, the spontaneous emulsification solvent diffusion does not require the use of high shear forces to produce PLGA nanoparticles. It has been mentioned that using this type of emulsion technique usually results in particles within the size range of 200 nm to 300 nm[44].

In addition to spontaneous emulsification solvent diffusion methods, other approaches that do not use high shear force to produce nanoparticles have also been reported, such as cryopreparation modification of the emulsion technique[37]. The temperature of the internal aqueous phase that contains the DNA is lowered to freezing point in order to obtain solid particles. This has been shown to be able to preserve the DNA structure from the effects of high shear forces. Other approaches include the condensation of DNA with cationic polymers in the internal phase.

More recently, a modified technique has been developed which is based on solvent diffusion and solvent displacement. This technique allows obtaining nanoparticles composed of PLGA blended with poloxamers/poloxamines polymers. The effect of this blending

technique on particle size, loading efficiencies and release profile has been studied in some details which are described below.

Csaba *et al,* have designed a new vector which has a potential application for DNA encapsulation, with controlled and continuous release features. Their work has been mainly focused on how to overcome the problems associated with DNA encapsulation in PLGA nanoparticles (i.e. structural and biological activity of DNA). In this system, they incorporated amphiphilic polymers which have the ability to interact with DNA by hydrogen bonds, and which can also protect DNA from degradation, thereby facilitating the gene expression[45, 46], (see Figure 1-9 in chapter one).

One of the most effective polymers used in this study was the group of polyoxyethylene derivatives (poloxamers and poloxamines)[47].One of the advantages of this system is that it provided an optimal condition for DNA encapsulation and protected the DNA from the acidic climate during degradation of PLGA nanoparticles. Equal amounts of poloxamers or poloxamines have been mixed with PLGA polymer to prepare nanoparticles by an emulsion-diffusion-evaporation technique. This technique uses steric stabilisation with ethylene and propylene oxides of the polymer backbone rather than the addition of extra surface active agents to stabilise the emulsion droplets.

Also, characterisation of PLGA blended nanoparticles, formed with poloxamers or poloxamines for size, zeta potential, encapsulation efficiency and biological activity have been studied.

The result showed that the size of nanoparticles blended with poloxamers were around 163±5 nm with PI (polydispersity) of 0.135 and 185±6 nm with PI 90.195 for Pluronic F68 and L121 respectively, on the other hand, the size of nanoparticles made with poloxamine

(tetronic 908) were 174±5 nm with PI = 0.271 and (tetronic 904) 168±9 nm with PI = 0.179.

Zeta potential showed highly negatively charged surface particles, for (Pluronic F68) - 0.43±6.4 mV, Pluronic L121 - 30±8 mV, whilst for (tetronic 908) - 26.9 ± 1.2 mV and (tetronic 904) - 38±3.3 mV.

For encapsulation of plasmid DNA and characterisation of loading capacity of the particles, the model of plasmid encoding green fluorescent protein (pEGFPs) was used. Their results after theoretical loading of 0.4% DNA showed the encapsulation efficiency of 35.2±13.2% for Pluronic F68 and 31.3±3.8% for Pluronic L121. On the other hand the data for tetronic 908 was 32.2±3.7% and for tetronic 904, 44.1±4.3%, in terms of encapsulation efficiencies (i.e. the amount of DNA loaded into particles compared to that in solution.

It was also suggested that the presence of certain surface active agents, such as Tween80 or poly (vinyl alcohol), will have positive effects on the encapsulation of hydrophilic DNA molecules in a hydrophobic polymer matrix. This effect may be understood by their interaction with DNA that could make it more hydrophobic, and this could facilitate the incorporation of the plasmid into the particle matrix.

Another interesting point which they have shown in the study by Alanso is that the presence of amine groups in the poloxamines structure (which have the ability to be protonated at ambient pH) is thought to facilitate interaction with DNA.

Also, high encapsulation efficiency in tetronic PLGA blends might be attributed to the combination of hydrophobicity and electrostatic interaction.

Despite the introduction of different approaches that have been used to develop nanoparticulate gene delivery systems, the design of an effective gene delivery system remains a challenge, and currently-developed systems also require further optimisation to produce nanoparticles highly loaded with DNA. Likewise, surface modification of the particles is needed to increase stability and prolong circulation time. In an attempt to produce an optimised gene delivery system, we have proposed a new blended system based on PLGA and another hydrophilic polymer, Jeffamine (Poly (ethylene glycol) bis (3-aminopropyl) terminated), to facilitate high encapsulation efficiency whilst improving the release profile. Moreover, we have also surface-coated the PLGA blended particles to highly hydrophilic polymers to provide stability with a view to obtaining longer circulation upon intravenous injection.

2.1. Aims and Objectives

The overall aim of this chapter is to focus on the development of a novel nanoparticulate gene delivery system to enhance encapsulation and improve release profile of DNA; using a combinatory approach to fabricate blended-matrix PLGA and surface functionalised nanoparticles.

Objectives and Hypotheses:

Objective one: To fabricate blended PLGA: Jeffamine nanoparticles using double emulsion technique, loading DNA on the blended particles and to characterise releasing profile.

Hypothesis: The addition of slightly positive charged excipient polymers (e.g. Jeffamine®) into PLGA based nanoparticles can improve encapsulation efficiency and release profile of biomolecules such as DNA.

Objective two: To coat the surface of blended PLGA: Jeffamine nanoparticles with amphiphilic block copolymers using conventional physical adsorption technique.

Hypothesis: the hydrophilic chains of coated block copolymers on the surface of PLGA: Jeffamine nanoparticles enhance colloidal stabilisation by providing steric "stealth" properties.

2.2. Materials and Methods

2.2.1. Instrumentation

Poly (lactide-co-glycolide) molar ratio 50:50 from Boehringer Ingelheim (Mw~11,000Da), Poly (ethylene glycol) bis (3-aminopropyl) terminated (Mn 1500 Da, Sigma-Aldrich), and Calf thymus DNA purchased from Invitrogen (average size of ≤ 2000 bp, concentration 10mg/ml in DNase-free, RNase-free distilled deionised water). Methylene chloride (HPLC grade), ethanol (absolute, for HPLC, ≥99.8% (Sigma-Aldrich), and DNase, RNase-FREE water (MP Biomedicals, LLC).

PDMAEMA-b-PEGMA$_{475}$ (Mn = 32.5 kDa and 1.3) was prepared and supplied by Dr. Wenxin Wang at NUI Galway.

2.2.1.1. Dynamic light scattering (DLS)

Values of the mean hydrodynamic radius of the nanoparticles were determined via scattered light recorded at 90° angle to incident radiation in Dynamic Light Scattering (DLS)) using a Viscotec Model 802 instrument equipped with an internal laser (825-832 nm) with a maximum radiation power of 60 mW.

Description of dynamic light scattering (DLS): when light hits small particles, the light scatters in all directions. Using a laser as the source of light (which is monochromatic and coherent) one can monitor the time-dependent fluctuation in the scattering intensity. The fluctuations are owing to the fact that small molecules or particles in the solutions are undergoing random movements which is named as "Brownian motion". The distances between the particles or molecules are changing constantly with time.
Therefore, the intensity of scattered light is also changing with time. This can be used to obtain information about the times scale movements of particles as follows:
With the assumption that the particles are spherical and non-interacting, the mean radius (R_H) is obtained from the Stokes-Einstein equation:

$$R_H = K_b T / 6\pi\eta D$$

Where R_H is the hydrodynamic radius, k_b is the Boltzmann constant, T is the temperature and η is the viscosity of the solvent. The speed of movement is inversely proportional to particle size (the smaller the particles are, the faster they move, or diffuse), and the speed can be detected by analysing the time dependency of the light intensity

fluctuations scattered from the particles when they are illuminated with a laser beam.

To determine the particle size, the samples were diluted with the same medium in which the particles are suspended (water or buffer), filtered, and at least five measurements of each sample taken. The mean diameter size and standard deviation were calculated. Data processing was performed with the software program OmniSize2.

2.2.1.2. Zeta Potential

Zeta potential (ξ) measurements of the nanoparticles were performed by laser Doppler anemometry using a Malvern Zetasizer 2000 equipped with a 10 mW He-Ne laser operating at a wavelength of 633 nm. Measurements were performed at 25 ± 0.10 C, on samples appropriately diluted with the medium described each time. Measurements were also taken in low ionic strength buffer, 1 mM PBS buffer adjusted to pH 7.4. The mean value and standard deviation for each sample was calculated from at least five measurements.

2.2.1.3. Transmission Electron Microscope

Morphology of the particles was examined using Transmission Electron Microscope (TEM) (Jeol Jem 1010 electron microscope, Japan). A sample of particle suspension was diluted with a solution of phosphotungstic acid (3% w/v, pH 7.4) and observed under TEM. One drop of sample was placed for one minute on a copper grid coated with a formvar carbon film. The excess of sample was wicked away with the aid of filter paper prior to imaging by TEM.

2.3. Polymer Synthesis

A Series of polymers and copolymers based on PLA, PLGA and PEGMA were synthesised and labeled from P1 - P8 (see table 3-I in chapter three). In this chapter only P4 and P8 are mentioned.

2.3.1. Synthesis of BSTSE-PLGA polymer (P 4)

BSTSE-PLGA, Poly (DL-lactide-co-glycolide) was synthesised by ROP (ring opening polymerisation) using 2-(benzylsulfanylthiocarbonylsulfanyl) ethanol (BSTSE) as initiator. First, DL-lactide (2.1g, 0.015 moles), glycolide (1.7g, 0.015 moles), and BSTSE (0.15g, 0.0005 moles) were added to a polymerisation tube and purged with argon several times before being heated in an oil bath to 80 °C and purged with argon for a further two hours. The reaction mixture was then heated to 140 °C in order to conduct the polymerisation in the melt phase. At this point, tin (II) (2-ethylhexanoate) (0.022g, 0.00005 moles) was added, and the mixture was stirred for 24 hours. The polymerisation tube was then removed from the oil bath and cooled down to room temperature. The polymer was recovered by dissolution in THF and precipitated in methanol (this was repeated three times to remove unreacted monomer).

The precipitate was filtered and dried under reduced pressure to yield a brown-yellow BSTSE-PLGA product (yield 92% weight of product). The molecular weight of the polymer and polydispersity index was determined by gel permeation chromatography using chloroform as eluent.

2.3.2. Synthesis of PLGA-PEGMA co-polymer (P 8)

The PLGA-PEGMA block copolymers were prepared by RAFT polymerisation. BSTSE-PLGA (1.2g, 0.0001 moles), Poly (ethylene glycol) methyl ether methacrylate (Mn 475 1.35g, 0.002 moles) and AIBN (0.003g, 0.000018 moles) were added into a round bottom flask, the flask purged with argon several times, followed by addition of anhydrous THF (5ml) under an argon atmosphere. The polymerisation mixture was allowed to stir until the components were completely dissolved. The mixture was then heated in an oil bath to 80 °C and stirred for 24 hours. The polymerisation product was dissolved in THF and reprecipitated three times in methanol, and the filtrate was dried under reduced pressure (yield 80%). The product was characterised by ^{1}H-NMR. GPC with chloroform as the eluent was used to calculate the molecular weight and polydispersity of the final block copolymers. This polymer was used for coating the NP1 nanoparticles (see section 2.4.3)

2.4. Particle preparation

2.4.1. Particle preparation of blank PLGA (uncoated) nanoparticles (NP1)

PLGA nanoparticles were prepared by double emulsion technique as mentioned elsewhere[48], using commercially available PLGA. Firstly, PLGA (11 kDa, 50:50 ratio) (100 mg) was dissolved in (2 mL) methylene chloride (DCM) and stirred gently until completely dissolved.

This organic solution was then mixed by vortex agitation for 30 s with a small aqueous phase (200 µl). Then, the obtained emulsion was poured onto a polar phase (25 ml ethanol) under moderate magnetic stirring, leading to immediate polymer precipitation in the form of

nanoparticles. The formulations were diluted with 25 ml of milli-Q water and the stirring was maintained for 10 minutes more. After solvent evaporation under vacuum at 30 °C (Rotavapor, Büchi R-114, Switzerland) nanoparticles were collected and concentrated in 1mM PBS buffer. The size distribution of the different nanoparticles was analysed by dynamic light scattering (DLS), surface charge was measured by Malvern Zetasizer 2000 and the morphology of the particles was analysed by Transmission Electron Microscope.

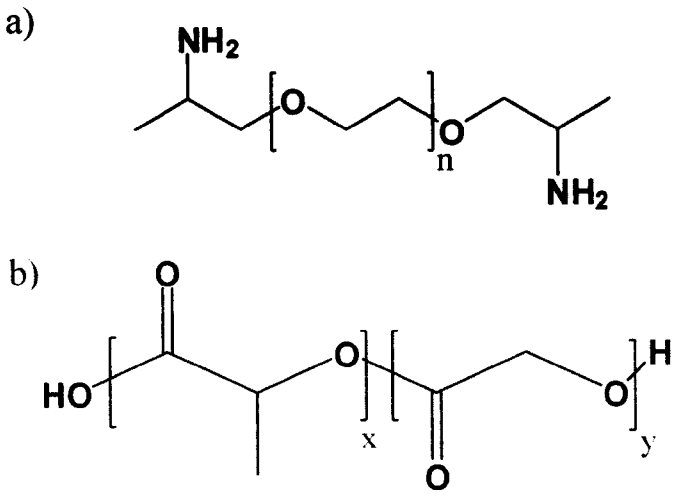
2.4.2. Particle preparation of blended PLGA: Jeffamine nanoparticles (NP2), loaded with Calf Thymus DNA (NP3)

The Calf Thymus loaded PLGA: Jeffamine blended nanoparticles (NP3) were prepared by double emulsion. First, PLGA (50 mg) and 5Jeffamine (50 mg) (PEG bis-aminopropyl) were dissolved in methylene chloride (2 mL) and stirred gently until completely dissolved.
Aqueous solution of DNA (200µl of 1mg/ml) was added to the polymer solution (0.4% of DNA to the total amount of PLGA) and mixed by vortex agitation for 30 seconds (primary emulsion formed).
The primary emulsion so obtained was poured onto a polar phase (25 ml Ethanol) under moderate magnetic stirring which resulted in immediate polymer precipitation in the form of nanoparticles. The formulations were diluted with 25 ml of 1mM PBS buffer and the stirring was maintained for 10 minutes more.

After solvent evaporation under vacuum at 30 °C (Rotavapor, Büchi R-114, Switzerland) nanoparticles were collected and concentrated in 1mM PBS. For the complete elimination of adsorbed Calf Thymus DNA, nanoparticles were extensively washed by a two-step procedure consisting of gel filtration (PD-10 desalting column with Sephadex G-25, Amersham Biosciences, Spain) and of particle

isolation/resuspension by centrifugation (15 min, 8000 RCF, 10 °C, Beckman J2-21 centrifuge). Blank PLGA: Jeffamine nanoparticles (NP2) were prepared using the steps above and using only DNAase free water instead of Calf Thymus DNA solution.

The size distribution of the different nanoparticles was analysed by dynamic light scattering (DLS), surface charge was measured by Malvern Zetasizer 2000 and morphology of the particles were analysed by Transmission Electron Microscopy.



Polymer structures of a) Jeffamine (Poly (ethylene glycol) bis (3-aminopropyl) terminated, b) PLGA poly (lactic-co-glycolic acid)

2.4.3. Adsorption of PLGA-PEGMA$_{475}$ block copolymer on the surface Blended PLGA: Jeffamine nanoparticles (NP4)

Nanoparticles were prepared using method described in section 2.4.2 in Chapter 2 (preparation of NP3). Except that the obtained formulations were diluted in PLGA-PEGMA$_{475}$ (P8) diblock copolymer (1% w/v) and incubated overnight under moderate stirring. For the complete elimination of free PLGA-PEGMA$_{475}$ diblock copolymer, nanoparticles

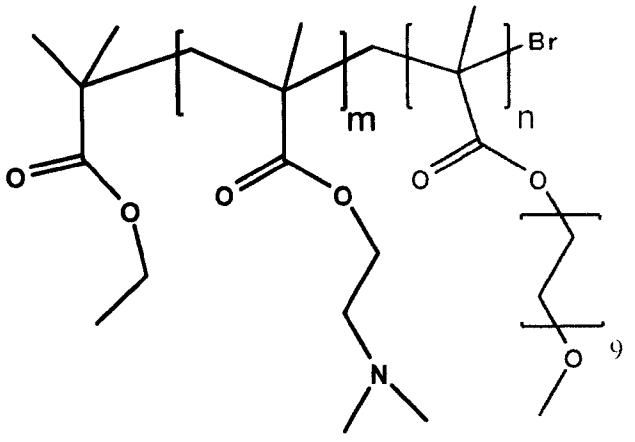
were extensively washed by a 2-step procedure consisting of gel filtration (PD-10 desalting column with Sephadex G-25, Amersham Biosciences, Spain) and of particle isolation/resuspension by centrifugation (15 min, 8000 RCF, 10 °C, Beckman J2-21 centrifuge).

The size distribution of the different nanoparticles was obtained by dynamic light scattering (DLS), surface charge was measured by Malvern Zetasizer 2000 and morphology of the particles was analysed by Transmission Electron Microscopy.

2.4.4. Adsorption of pDMAEMA-PEGMA$_{475}$ block copolymer on the surface PLGA: Jeffamine blended nanoparticles (NP5)

Nanoparticles were prepared by the method as mentioned in (section 2.4.2) for (NP3). But the obtained formulations were diluted in PEGMA-PDAEMA diblock copolymer (1% w/v) and incubated overnight under moderate stirring.
For the complete elimination of free PEGMA-pDAEMA (this diblock copolymer was supplied by NUI galway) diblock copolymer, nanoparticles were extensively washed by a 2-step procedure consisting of gel filtration (PD-10 desalting column with Sephadex G-25, Amersham Biosciences, Spain) and of particle isolation/resuspension by centrifugation (15 min, 8000 RCF, 10 °C, Beckman J2-21 centrifuge).

The size distribution of the different nanoparticles was analysed by dynamic light scattering (DLS), surface charge was measured by Malvern Zetasizer 2000 and morphology of the particles was analysed by Transmission Electron Microscopy.



Structure of polymer pDMAEMA-b-PEGMA475

2.4.5. Determination of plasmid DNA encapsulation efficiency

The theoretical loading of Calf DNA was 0.4% (w/w) in regards to the total amount of PLGA in the nanoparticles. For the determination the actual amount of encapsulated cDNA, encapsulation efficiency was calculated from the amount of the free Calf Thymus DNA present in the aqueous phase of the formulation (obtained by centrifugation without particle washing). The amount of this Calf Thymus DNA was determined by spectrophotometry (UV-Visible Spectrometer, UV-1603 Shimadzu, Spain) using standard calibration curve of Calf Thymus DNA in DNAase free water at 260 nm.

2.4.6. Release profile of plasmid DNA from the nanoparticles

The *in vitro* release of cDNA from blended PLGA: Jeffamine nanoparticles (NP4) were investigated as follows. Nanoparticles (10 mg) were suspended in PBS buffer (1.5 ml, 1mM) at 37 °C under continuous agitation. At various time intervals, the supernatant was withdrawn and fresh buffer was replenished. The amounts of Calf Thymus DNA in

The supernatant were determined by UV spectrophotometry at 260 nm. The experiments were performed in triplicate.

2.5. Results and Discussion

2.5.1. Polymers syntheses and characterisation (P 4 and P 8)

Series of PLA, PLGA polymers and PLGA-PEGMA block copolymers were prepared by combining ring opening polymerisation (ROP) and reversible addition fragmentation transfer (RAFT) and referred to as P1 to P8 (chapter 3). However, in this chapter only the syntheses of BSTSE-PLGA (P4) polymer and PLGA-PEGMA$_{475}$ copolymer (P8) (which latter was physically adsorbed on blended PLGA: Jeffamine nanoparticles) are described.

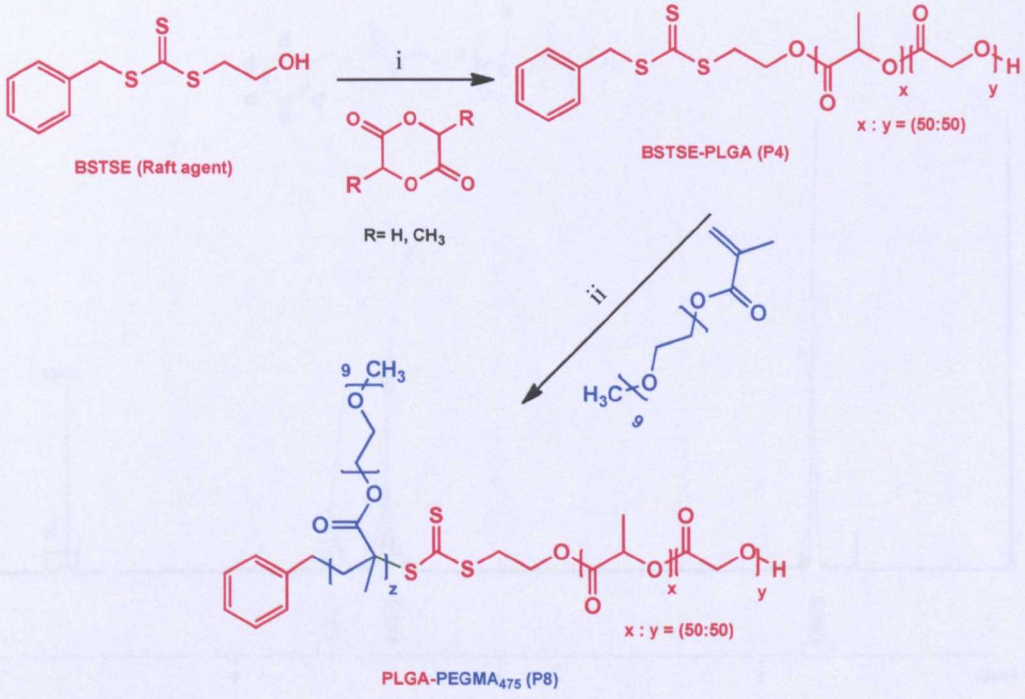


Figure 2-1. Syntheses of Polymers (P4 and P8), (i) Sn (II), 140 °C, 24 h, (ii) THF, AIBN, 80 °C 24 h, (see Materials and Methods section)

The properties of BSTSE-PLGA (P4) and PLGA-$PEGMA_{475}$ (P8) copolymers synthesised by ROP and RAFT are summarised in table 2-I, describing the molecular weight and molecular weight distributions based on results obtained from gel permeation chromatography.

Table 2-I. Characteristics of PLGA-PEGMA475 diblock copolymer synthesised by ROP and RAFT process

Entry	Polymer	[a]*Mn* (theo)*10^3	*Mn* (GPC)*10^3	*Mw* (GPC)*10^3	*Mw/Mn*
P 4	BSTSE-PLGA	9.6	13.3	20.1	1.5
P 8	PLGA-PEGMA	27	27.1	48.1	1.7

[a] was calculated by multiplying the conversion with the targeting molecular weight.

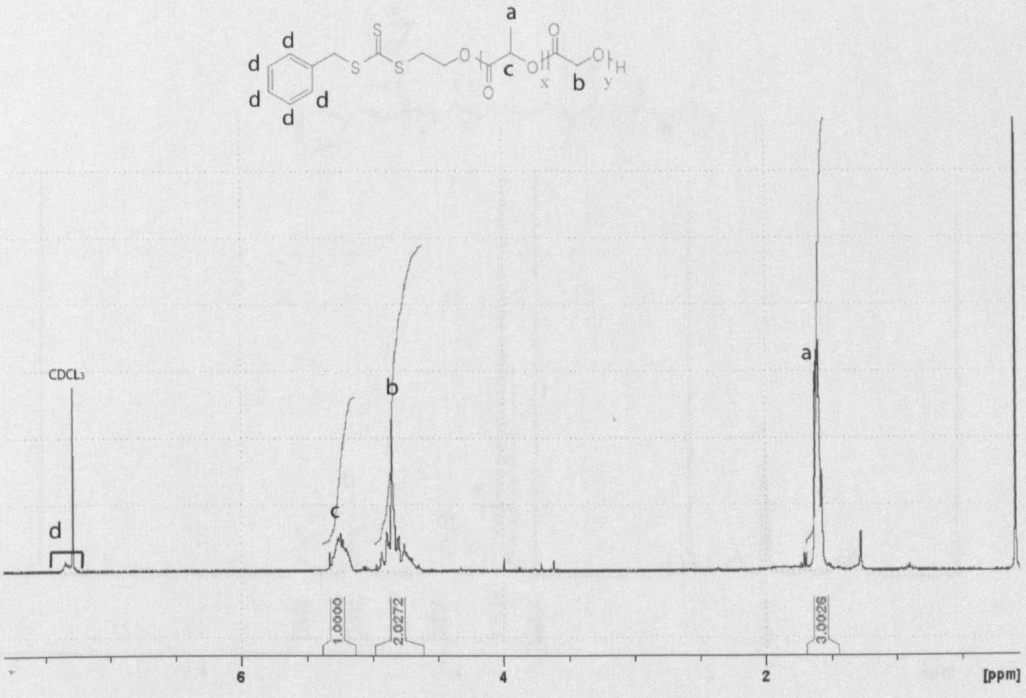


Figure 2-2. ^{1}HNMR spectrum of BSTSE-PLGA P 4 with Mn (GPC) =13.3 K, PDI= 1.5 in $CDCl_3$.

The structure of the PLGA polymer end-capped with BSTSE was also confirmed from ^1H NMR spectrum obtained in ($CDCl_3$). The peaks which are labelled in Figure 3-4 referred to the protons of the backbone of PLGA polymer (a-c) and the end group for BSTSE (d). δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H, CH2- C=O), 1.58 (d, 3H, CH_3-CH), 7.3-7.4 (m, 5H, Ph).

Owing to the low intensity of the phenyl proton adsorptions compared to the protons of the PLGA in the ^1H NMR spectrum, it was difficult to calculate the molecular weight via ^1H-NMR alone, however Using the integration on the spectrum, it was possible to confirm the 50:50 ratio of lactic to glycolic acid units in the polymer which was the target in the synthesis of the polymer.

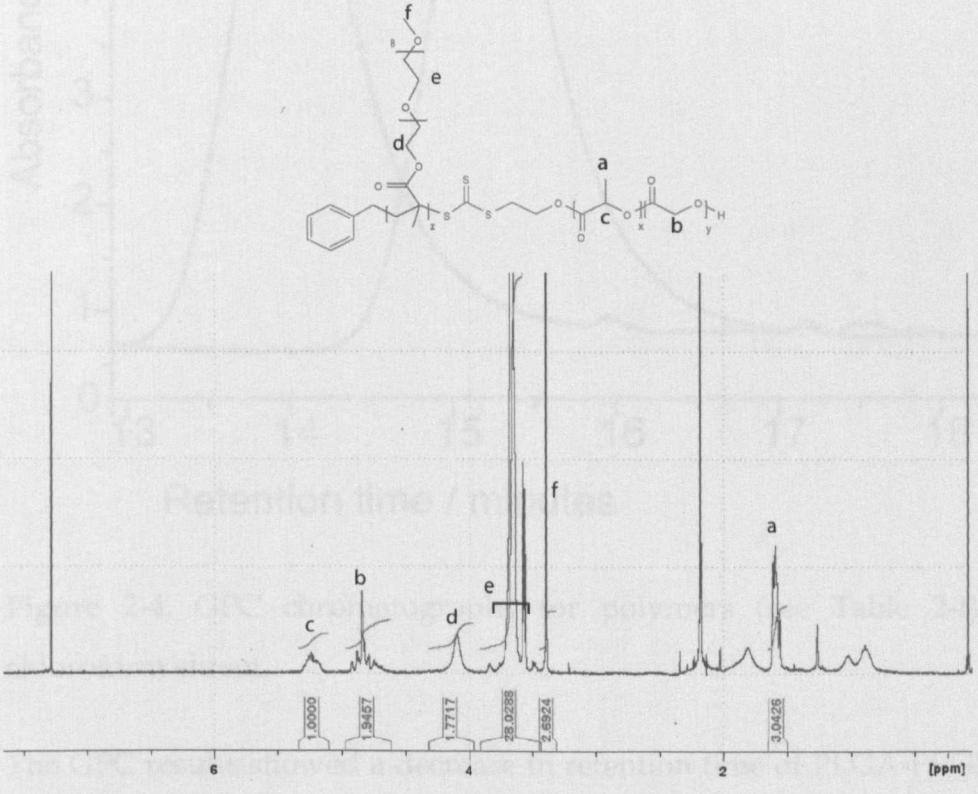


Figure 2-3. ^1HNMR spectrum of PLGA-PEGMA P 8 with Mn (GPC) =2.7k, PDI= 1.5 in $CDCl_3$.

From the ^1H NMR spectrums of PLGA-PEGMA$_{475}$ block copolymer, it was possible to characterise the structure by assigning the peaks as shown in (Figure 3-5). The peaks labelled from e-g were assigned to the protons on the PLGA block. The peaks labelled (h) were assigned to PEGMA units.

δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H, CH_2- C=O), 3.94-4.14 (m, CH_2, CH_2-O- C=O), 3.4-3.8 (m, CH_2CH_2O), 1.58 (s, 3H, CH3), 1.6-1.9(m, CH_2), 0.7-1.3 (s, CH3).

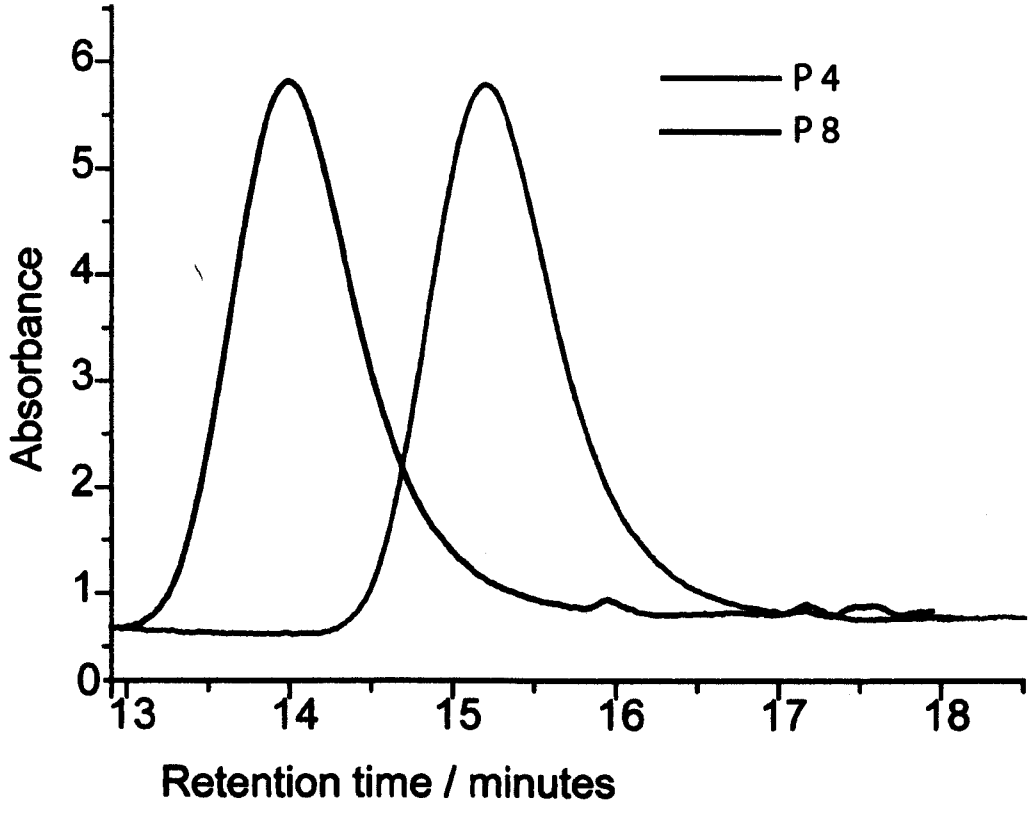


Figure 2-4. GPC chromatographs for polymers (see Table 2-I) in chloroform eluent.

The GPC results showed a decrease in retention time of PLGA-PEGMA in comparison to the GPC curve of the first block (BSTSE-PLGA). This was a preliminary indication that the second block (PEGMA) was inserted to the polymer chain between the trithiocarbonate unit and PLGA.

2.5.2. Fabrication of nanoparticles and characterisation

Conventionally, PLGA nanoparticles have been prepared by double emulsion technique W/O/W (water in oil in water) to encapsulate hydrophilic molecules such as DNA and protein. High shear forces (e.g. homogenisation or sonication) that have been utilised to obtain particles sizes in the nanometre size range have been reported to have a negative effect on the integrity of the molecules (shear-induced degradation) and resulted in compromising their biological activity.

Recently, Csaba *et al*[46], have introduced a modified double emulsion technique to encapsulate plasmid DNA under a mild conditions in which nanoparticles are formulated without using any shear forces. In addition to a modified double emulsion technique, they have also prepared blend nanoparticles composed of PLGA and PEO derivatives using the modified emulsion technique. Mixing of PEO with PLGA polymer in the initial steps of the emulsion technique was believed to provide a protecting environment for DNA during nanoparticle preparation and release.

However, these types of nanoparticle showed low encapsulation efficiency and lacked the stealth properties which are necessary to prolong circulation time and prevent early elimination from blood MES.

Based on these requirements, in this chapter of the thesis, a new nanoparticle composition was formulated which was composed of PLGA blended with Jeffamine. PLGA: Jeffamine blended nanoparticles were compared to classical PLGA nanoparticles. The DNA encapsulation efficiency of this new nanoparticle composition was also studied in addition to adsorption of two different amphiphilic block copolymer on the surface of the blended nanoparticle to formulate "core-shell" nanoparticles.

2.5.2.1. Fabrication of PLGA nanoparticles (NP1, NP2)

Physicochemical characteristics (particle size, surface charge and morphology) were studied for two different types of nanoparticle formulation, PLGA nanoparticles NP1 and PLGA: Jeffamine blended nanoparticles NP2. These two formulations were different mainly in their matrix composition; the matrix of NP1 was only composed of PLGA polymer whereas the matrix of NP2 contained a mixture of PLGA and Jeffmaine polymers. Both types of nanoparticles were formulated using double emulsion technique (schematic illustration of this technique is shown in Figure 2-5).

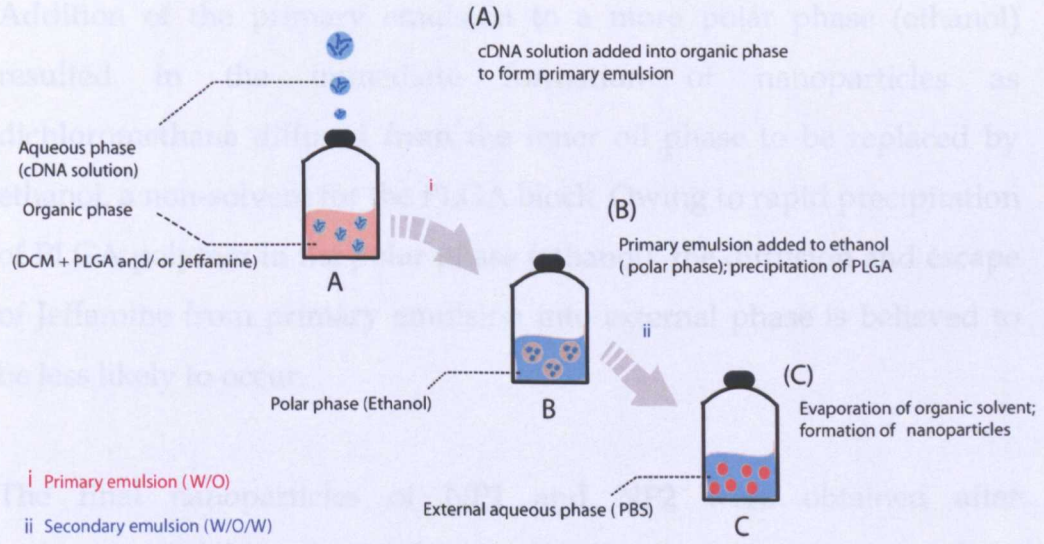


Figure 2-5. Preparation of nanoparticles using double emulsion technique.

In (A) cDNA in water is added into dichloromethane (DCM) containing either PLGA alone or with Jeffamine.

Addition of this primary emulsion to ethanol (B) results in collapse of the hydrophobic core as the DCM partitions into the bulk phase. Solvent evaporation (C) yields the final nanoparticles.

In order to compare the properties of PLGA based nanoparticles and PLGA: Jeffamine blended nanoparticles a double emulsion technique was utilised as illustrated in Figure 2-1. To accomplish this, an aqueous solution (in this case only PBS buffer solution was used) was added into dichloromethane (organic phase) containing only PLGA (NP1) or PLGA and Jeffamine (NP2). A mild vortex was needed to form the primary emulsion which consisted of water droplets suspended in the organic phase of DCM. Owing to the hydrophilic nature of Jeffamine, it is most likely that some of the polymer was around the water droplets. Addition of the primary emulsion to a more polar phase (ethanol) resulted in the immediate formation of nanoparticles as dichloromethane diffused from the inner oil phase to be replaced by ethanol, a non-solvent for the PLGA block. Owing to rapid precipitation of PLGA polymer in the polar phase (ethanol), the diffusion and escape of Jeffamine from primary emulsion into external phase is believed to be less likely to occur.

The final nanoparticles of NP1 and NP2 were obtained after evaporation of the organic solvent under the same conditions and these particles were of low size polydispersity, but the surface charges were slightly different. In NP1 nanoparticles the zeta potential was slightly higher presumably due to surface negative charges owing to a high presence of PLGA carboxylic groups on the surface (Figure 2-8). Whereas in NP2 the zeta potential value was slightly less negative which may have been because some of Jeffamine polymers (which carry protonated amines) were localised at the surface and masking the negative carboxylic groups of the PLGA polymer.

Several batches of nanoparticles were prepared via this double emulsion technique and their size distributions are shown in Figure 2-6.

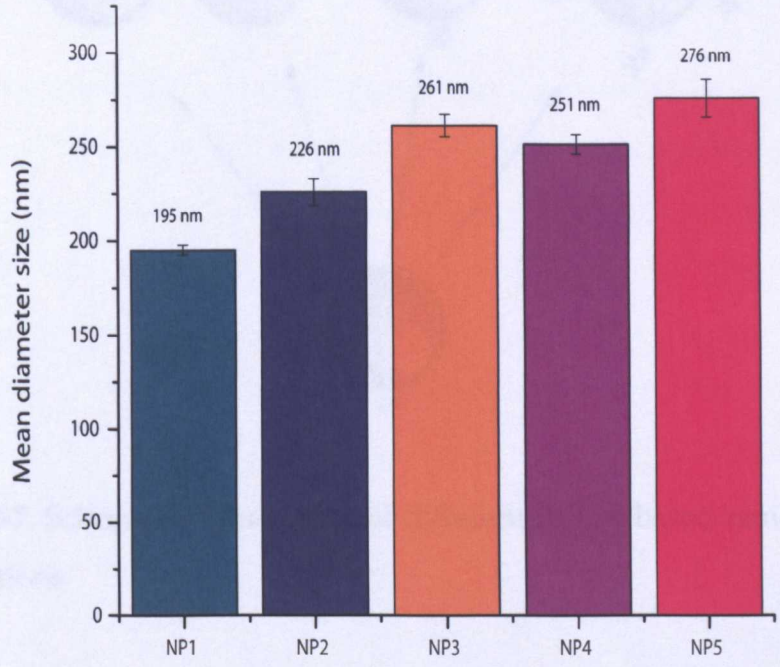


Figure 2-6. Mean Particle Size of nanoparticles prepared by double emulsion technique: NP1 represent PLGA nanoparticles, NP2 nanoparticles composed of PLGA: Jeffamine blend, NP3 represent cDNA loaded NP2 nanoparticles, NP4 and NP5 represent NP2 nanoparticles surface modified with PLGA-PEGMA475 and PEGMA-pDMAEMA diblock copolymers respectively.

As shown in Figure (2-6) the mean size of NP2 was slightly larger than NP1 although both were prepared via the same method and under the same conditions, ($p = 0.629$, there is no significant difference between the sizes of NP1 and NP2). The difference in mean size may have resulted from incomplete mixing of hydrophilic Jeffamine and hydrophobic PLGA in the matrix and also Jeffamine polymers were

more likely to have entrapped some water molecules in the matrix of the nanoparticles resulting in a slight increase in the size of NP2.

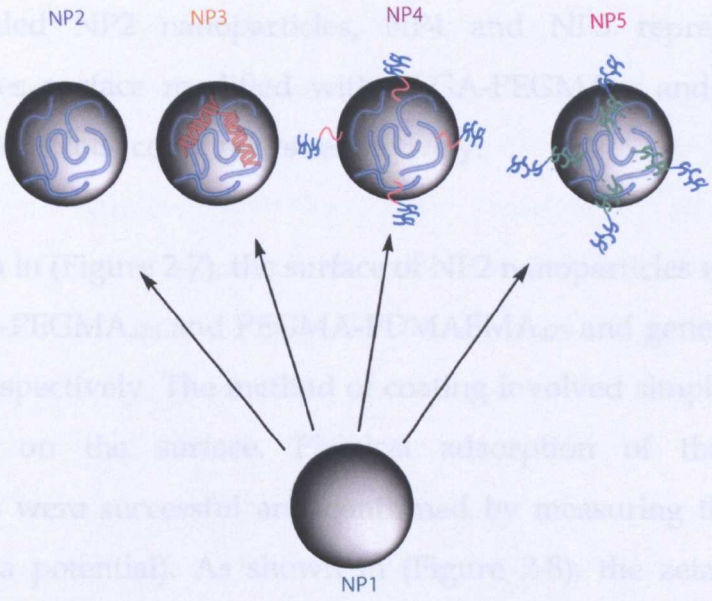


Figure 2-7. Schematic illustration of different PLGA based nanoparticle formulations.

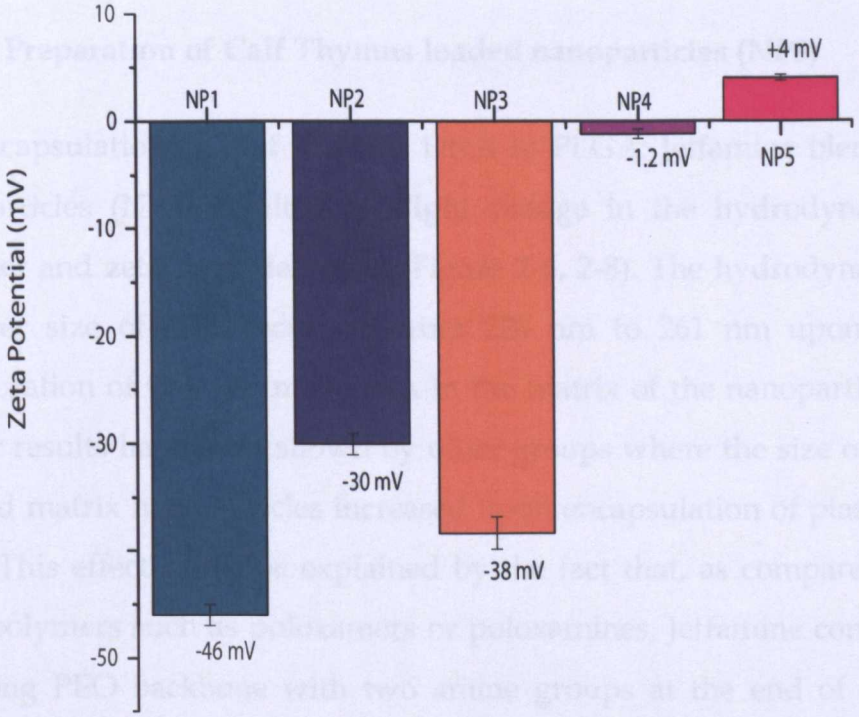


Figure 2-8. Mean zeta potential values of nanoparticles prepared by double emulsion technique: NP1 represents PLGA nanoparticles, NP2 nanoparticles composed of PLGA: Jeffamine blend, NP3 represent cDNA loaded NP2 nanoparticles, NP4 and NP5 represent NP2 nanoparticles surface modified with PLGA-PEGMA$_{475}$ and PEGMA-pDMAEMA diblock copolymers respectively.

As it shown in (Figure 2-7), the surface of NP2 nanoparticles was coated with PLGA-PEGMA$_{475}$ and PEGMA-PDMAEMA$_{475}$ and generated NP3 and NP4 respectively. The method of coating involved simple physical adsorption on the surface. Physical adsorption of the diblock copolymers were successful and confirmed by measuring the surface charge (zeta potential). As shown in (Figure 2-8), the zeta potential values changed from -30 mV to near zero owing to the formation of hydrophilic shell of PEGMA polymer chains around the surface of the nanoparticles.

2.5.2.2. Preparation of Calf Thymus loaded nanoparticles (NP3)

The encapsulation of Calf Thymus DNA in PLGA: Jeffamine blended nanoparticles (NP3) resulted in slight change in the hydrodynamic diameter and zeta potential value (Figure 2-6, 2-8). The hydrodynamic diameter size of NP2 increased from 226 nm to 261 nm upon the incorporation of Calf Thymus DNA in the matrix of the nanoparticles. Similar results have been shown by other groups where the size of the blended matrix nanoparticles increased upon encapsulation of plasmid DNA. This effect could be explained by the fact that, as compared to other polymers such as poloxamers or poloxamines, Jeffamine consists of a long PEO backbone with two amine groups at the end of each chain. This particular characteristic of Jeffamine may lead to different kind of interaction with the negatively charged DNA, hence different

architectural organisation of Calf Thymus DNA and Jeffamine polymer may occur.

With regard to surface charge of Calf Thymus loaded PLGA: Jeffamine blended nanoparticles (NP3), a slight increase in zeta potential value has been observed comparing to unloaded PLGA: Jeffamine blended nanoparticles (NP2) after incorporation of the Calf Thymus DNA in the nanoparticles. Similar effects have been reported by other groups where the surface charge of the nanoparticles became more negative upon loading with Plasmid DNA. This result could be explained by the fact that the blended polymers may have different orientations owing to the presence of Calf Thymus DNA, in addition to that , during the addition of the primary emulsion to a more polar phase (ethanol), hydrophilic molecules of DNA in the aqueous droplet may diffuse from the droplet into external phase and some of these may entrap on the surface as a result of rapid precipitation of PLGA polymers in non-solvent ethanol during the solvent diffusion and particles formulation.

The combination of the PLGA and Jeffamine polymers with the double emulsion method resulted in efficient encapsulation of Calf Thymus DNA. The extent of incorporation was 84.4 % of the DNA in solution, corresponding to a theoretical loading of 0.4% with respect to the mass of polymer in the emulsion (~ 4ug DNA per mg of polymer).

Recovery of DNA from the nanoparticles following treatment of the dried copolymers with 0.5% N NaOH indicated that 3.5ug/mg of Calf Thymus DNA had been encapsulated as determined by UV spectroscopy and comparison to standard absorption values at 260 nm.

It must be noticed that cDNA was loaded on NP3 only and it was not incorporated into NP4 or NP5, as the latter nanoparticle systems were designed to test the surface fuctionalisation. These systems were tested separately to avoid the influence of surface fuctionalisation on encapsulation efficiency of cDNA and vice versa.

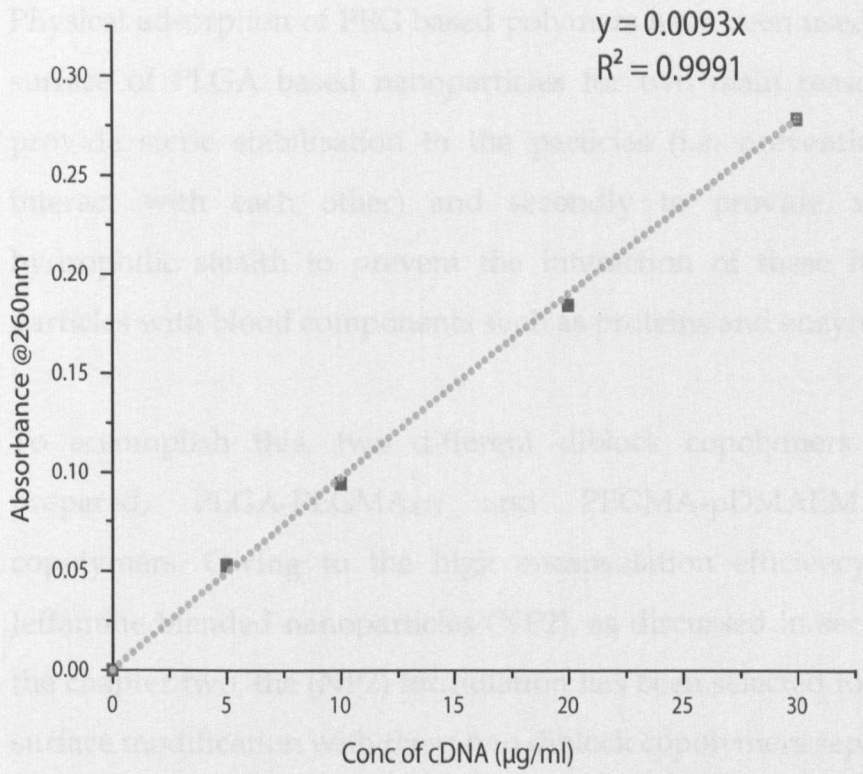


Figure 2-9. Calibration of Calf thymus DNA in water.

2.6. Surface modification of PLGA: Jeffamine blended nanoparticles with diblock copolymers (NP4 and NP5)

The negative surface charges of uncoated PLGA nanoparticles (NP1), PLGA: Jeffamine blended nanoparticles (NP2) and Calf Thymus loaded PLGA: Jeffamine nanoparticles (NP3) can provide weak colloidal stability through charge-charge repulsion mechanism. However, to make these particles suitable for intravenous administration, the surface of the particles should be coated with hydrophilic polymers such as poly (ethylene glycol). Uncoated particles are easily recognised by mononuclear phagocyte (MPS); this can be attributed to the hydrophobic properties of the particle forming polymers that favour the adsorption of blood components, which is a step before elimination from the blood.

Physical adsorption of PEG based polymers have been used to coat the surface of PLGA based nanoparticles for two main reasons, first to provide steric stabilisation to the particles (i.e. preventing them to interact with each other) and secondly to provide a protective hydrophilic stealth to prevent the interaction of these hydrophobic particles with blood components such as proteins and enzymes.

To accomplish this, two different diblock copolymers have been prepared, PLGA-PEGMA$_{475}$ and PEGMA-pDMAEMA diblock copolymers. Owing to the high encapsulation efficiency of PLGA: Jeffamine blended nanoparticles (NP2), as discussed in section 2.8.2 of the chapter two, the (NP2) formulation has been selected to be used for surface modification with these two diblock copolymers separately.

Using the double emulsion technique, after the formation of nanoparticles in the polar phase (ethanol), the particles were diluted with (1% w/v) solution of PLGA-PEGMA$_{475}$ or PEGMA-pDMAEMA diblock copolymers in 1mM PBS buffer solution. This resulted in the formation of coated nanoparticles NP4 and NP5 respectively.

Considering the amphiphilic nature of these two diblock copolymers, some of them may form micelles in aqueous solution. This has been also reported by another group thus further study is required to investigate how these polymers behave during the physical adsorption process. In a similar way the nanoparticles were subjected to the steps of washing, centrifugation and passing through PD-10 desalting column to remove unabsorbed diblock copolymers.

The mechanism of adsorption of PLGA-PEGMA$_{475}$ copolymers varies from PEGMA-pDMAEMA copolymer. The PLGA-PEGMA$_{475}$

copolymer consists of a hydrophobic block of PLGA polymer linked with a hydrophilic block of $PEGMA_{475}$, whereas the PEGMA-pDMAEMA copolymer consists of one positively charged block linked to a hydrophilic PEGMA block. Owing to the difference in nature of the diblock copolymers, their physical adsorption mechanism varies, as follows.

In the case of PLGA-$PEGMA_{475}$, the hydrophobic PLGA block anchors onto the surface of PLGA nanoparticles via hydrophobic interaction and allows the hydrophilic PEGMA block to be projected toward the aqueous medium, thus overlying the surface of the PLGA based nanoparticles. Whereas in the case of PEGMA-pDMAEMA, the pDMAEMA block, which is positively charged (owing the presence of amine groups), interacts with the negative surface charges of PLGA based nanoparticles, thus masking the surface of the nanoparticle via electrostatic interactions.

Surface coating of (NP2) nanoparticles with PLGA-PEGMA475 or PEGMA-pDMAEMA diblock copolymers have resulted in slight increase in the particle size but substantial decrease in negative surface charges (Figure 2-6, 2-8). The size of particles increased from 226 nm to 251 nm and 276 nm after the adsorption of PLGA-PEGMA475 and PEGMA-pDMAEMA respectively to generate (NP4) and (NP5) formulation.

The sizes of (NP4) were slightly smaller than those of (NP5), maybe owing to the fact that the positive charge of pDMAEMA can also attract more water molecules and thus expand the hydrodynamic diameter. In addition to that, the hydrophobic interaction of PLGA block with the surface of the nanoparticles is stronger than the electrostatic interaction of pDMAEMA, thus the polymer chains are more compacted on the surface.

In respect to surface charges after physical adsorptions of the diblock copolymers, the zeta potential value of (NP2) nanoparticles changed from - 30 mV to -1.2 mV (NP4) and +4 mV (NP5) for adsorptions of PLGA-PEGMA475 and PEGMA-pDMAEMA diblock copolymers respectively. The overlaying of the nanoparticles with these copolymers resulted in the formation of the so called "core-shell" structure.

The TEM micrographs of NP2, NP4 and NP5 (3% phosphotungstic acid negative staining) showed the particles to be spherical in shape and particle sizes determined from TEM micrographs were slightly smaller than the hydrodynamic diameters recorded by dynamic light scattering (DLS), most likely due to air-drying of the nanoparticles on the copper grid leading to collapse of hydrated ($PEGMA_{475}$) segments on the outer shells. (Figure 2-10).

The TEM micrographs of uncoated nanoparticles NP2 appeared to have higher polydispersity and the particles seemed to be aggregated upon drying on the copper grid. Meanwhile, the nanoparticles of NP4 and NP5 were of better defined spherical shape with low polydispersity and the nanoparticles were not aggregated upon drying on the grid. This particular characteristic of NP4 and NP5 can be explained by the fact that the surface of the particles were overlaid by PLGA-PEGMA475 and PEGMA-pDMAEMA diblock copolymers which provided steric stabilisation and thus prevented the particles from interacting with each other, hence no aggregation occurred.

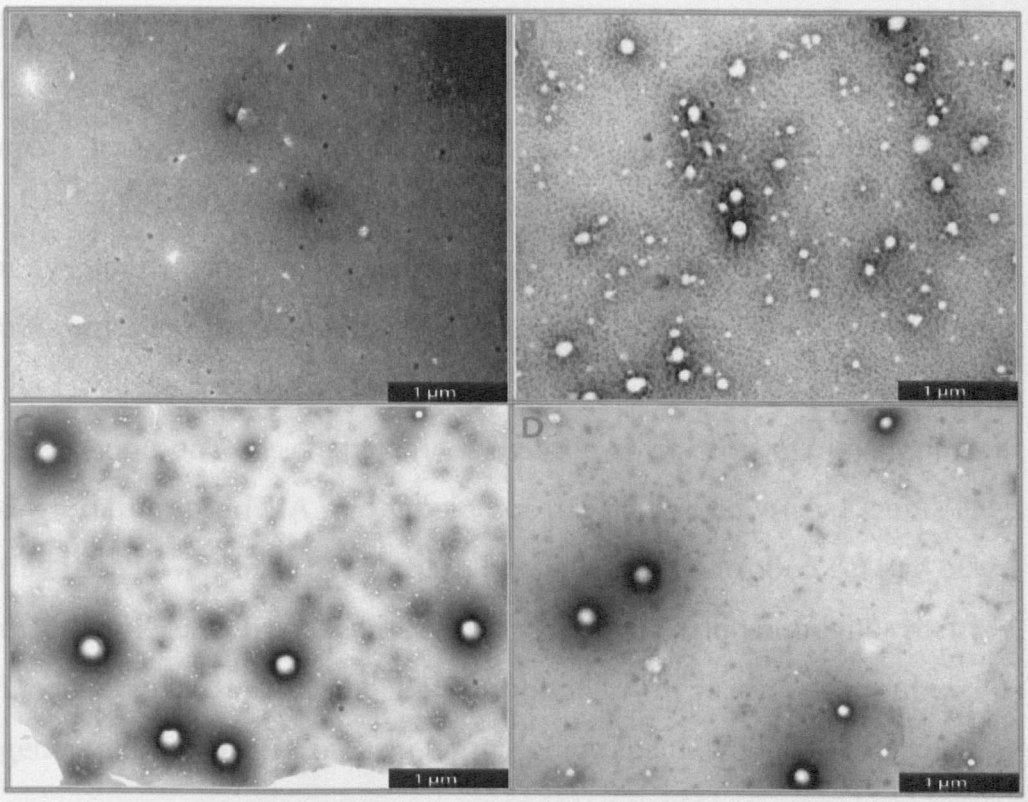


Figure 2-10. Transmission Electron Micrographs (TEM) of a) control (PBS buffer added onto the copper grid), b) NP2 of PLGA: Jeffamine blend), c) NP4 (NP2 nanoparticles surface modified with PLGA-$PEGMA_{475}$), d) NP5 (NP2 nanoparticles surface modified with PEGMA-pDMAEMA).

2.7. In vitro release profile of Calf Thymus DNA from nanoparticles (NP3)

Generally, the releases of hydrophilic molecules such as DNA or proteins from biodegradable nanoparticles follow a mechanism in which the degradation of the polymers leads to erosion of the matrix. This in turn results in formation of an aqueous channel through which the hydrophilic molecules are diffused out.

In conventional PLGA nanoparticles, the releases of encapsulated molecules (e.g. DNA, protein) follow a tri-phasic release pattern. This characterised by an initial burst release owing to the presence of loosely associated DNA with the surface. Surface localisation of the molecules may occur in the organic solvent evaporation phase which causes the molecules to escape towards the external medium.

The second phase is characterised by little or no release until significant degradation of PLGA and erosion of the matrix polymer occurs. Then this is followed by a third phase consisting of controlled release of the macromolecules from the matrix through generated aqueous channels. Upon degradation of the PLGA polymer, an acidic environment is generated which affects the integrity of encapsulated DNA or protein.

To overcome this problem, blended matrix nanoparticles consisting of PLGA polymer physically mixed with Jeffamine polymer have been prepared. The release profiles from the blend nanoparticles were investigated. The *in vitro* release experiment showed that incorporation of Jeffamine in PLGA nanoparticles has a significant effect on the release pattern of the encapsulated molecules.

As can be observed in (Figure 2-11), the blended matrix nanoparticles (NP3) provided continuous release of cDNA. Similar results have been observed by Csaba *et al,* in which different blended nanoparticles based on PLGA and PEO derivatives were prepared and the release of DNA investigated and shown to be of a similar pattern.

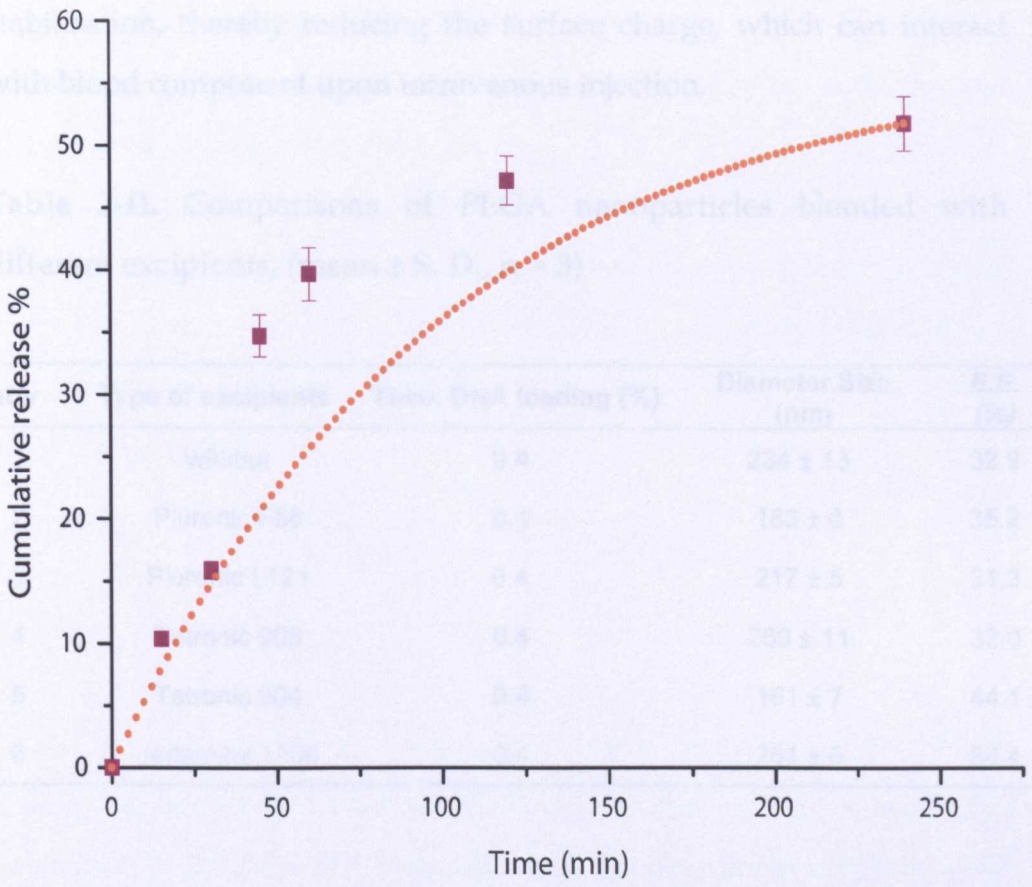


Figure 2-11. Cumulative release (%) of Calf Thymus DNA from nanoparticles (NP3)

2.8. Conclusion

In conclusion, it has been shown that it is possible to prepare PLGA nanoparticles with higher encapsulation efficiency and an improved release profile using a blend of PLGA with Jeffamine polymer, (up to 84.4% encapsulation of DNA compared to only 44.1% reported for blended PLGA: Tetronic 904 by Csaba *et al*), (table 2-II). The combination of double emulsion technique and this new blend system also generated particles within the nanoscale range. Moreover, the surface of the particles were modified with highly hydrophilic polymer

(PEG based polymer) in order to provide the particle system with steric stabilisation, thereby reducing the surface charge, which can interact with blood component upon intravenous injection.

Table 2-II. Comparisons of PLGA nanoparticles blended with different excipients, (mean ± S. D., n = 3)

Entry	Type of excipients	Theo. DNA loading (%)	Diameter Size (nm)	*E.E. (%)*
1	without	0.4	234 ± 13	32.9
2	Pluronic F68	0.4	183 ± 6	35.2
3	Pluronic L121	0.4	217 ± 5	31.3
4	Tetronic 908	0.4	269 ± 11	32.0
5	Tetronic 904	0.4	161 ± 7	44.1
6	Jeffamine 1500	0.4	261 ± 6	84.4

The advantage of incorporating Jeffamine polymer (Poly (ethylene glycol) bis (3-aminopropyl) terminated) over other amphiphilic copolymers such as poloxamers and poloxamines lies in the ability of this polymer to display accessible low positive charges at the end, which, perhaps, enable them to bind with DNA through electrostatic interaction. Owing to the good miscibility of Jeffamine within the hydrophobic matrix of PLGA nanoparticles, it was possible to achieve higher encapsulation efficiencies and an improved release kinetic. Therefore, PLGA blended nanoparticles can be introduced as potential carriers for DNA delivery systems.

Despite the successful results shown for higher encapsulation efficiency and improved release profile of DNA using blended PLGA: Jeffamine particulate system, there is still concern over surface fuctionalisation of the nanoparticles using physical adsorption of PEG-based block

copolymers. This type of physical surface coating is very likely to be displaced, for example by blood components upon intravenous injection, which may lead to disturbance of colloidal stability and eventually aggregation of the particles.

Therefore, there is a clear indication for the development of more advanced nanoparticulate system of core-shell type structure, using block copolymers, in which the outer shell is covalently linked to the core; hence the colloidal stability can be preserved upon mixing with other components.

Since the discovery of controlled living polymerisation (CLP) techniques, access to well-defined block copolymers has dramatically increased. To prepare PLGA based block copolymers that can be used to fabricate core-shell type nanoparticles, we have, therefore, focused on the synthesis of PLGA-PEGMA$_{475}$ type copolymers via combination of ring opening polymerisation (ROP) and reversible addition fragmentation transfer (RAFT). The advantage of this synthetic route lies in the flexibility of the system to obtain well-defined biocompatible block copolymers. The detail of synthesis PLGA based block copolymer is discussed in chapter three.

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Chapter 3

Synthesis of biodegradable and biocompatible co-polymer micelles

3. Introduction

Recently considerable interest has been paid to the synthesis of block copolymers with an amphiphilic nature. The difference in solubility of the hydrophobic to hydrophilic block leads to self-assembly in aqueous media to form polymeric micelles[1-3]. In the presence of water, the hydrophobic segments tend to decrease their contact with water, because of their insolubility, and are attracted toward each other. In the mean time, the hydrophilic segments separate from each other and interact more with water molecules to form an outer shell that covers the hydrophobic core[4].

The biomedical applications of these block copolymer micelles have rapidly expanded into novel carrier systems for targeting of poorly soluble drugs[5-7]. These polymeric micelles as drug delivery carriers offer several unique characteristics owing to the submicron size range, i.e. escape from renal excretion, passive targeting to solid tumours, and prolonged circulation in the blood stream. Moreover, they have fairly good thermodynamic stability in aqueous media due to the relatively low critical association concentration (CAC) [8-11]. Another advantage of polymeric micelles is their ability to encapsulate large amounts of insoluble drugs in the hydrophobic core.

The distribution of these polymeric micelles in the body may depend on their size and surface charge[12-14].

In this regard, the ability to control the size and surface charge of these carriers has a crucial effect on *in vivo* therapeutic efficacy[12, 14]. Critical micelle concentration (CMC) in these polymers can be defined as that concentration below which only single chains are present but above which both single chains and micellar aggregates can be found. Many techniques are available for the determination of critical micelle concentrations. In principle, one can use any physical property which depends upon the particle size or the number of particles. Such properties as the surface tension, electrical conductivity, osmotic pressure, interfacial tension, or light scattering as a function of concentration have been used for this purpose. Critical micelle concentrations can also be determined from the change in the spectral characteristics of some dye probes added to the surfactant solution[15-17].

Many amphiphilic block copolymer micelles being studied for drug delivery applications have a hydrophilic block to form the outer shell; perhaps in most cases the hydrophilic block consists of biocompatible poly (ethylene oxide)[18-20]. The presence of PEO blocks on the outer shell of the polymeric micelles has been shown to prevent opsonisation and subsequent recognition by macrophages of the reticuloendothelial system (RES)[21]. This, in turn, provides a prolonged blood circulation, which enables the micelles to localise passively in tissues and organs before being eliminated from the body.

The hydrophobic core of the micelles can be utilised as a reservoir for encapsulation of various therapeutic or diagnostic agents such as hydrophobic drugs[20].

Designing the core of the polymeric micelles with a biocompatible polymer is an extremely attractive option for drug delivery applications considering the safety of the system.

There are several biocompatible and/or degradable hydrophobic polymers that have been used to design the core of the polymeric micelles, among these polymers are poly (propylene oxide) (PPO)[22, 23], poly (β-benzyl L-aspartate) (PBLA)[24], poly (γ-benzyl-L-glutamate) (PBLG)[25], polycaprolactone (PCL)[26], poly (lactic acid) (PLA)[27], and poly (D, L-lactide) (PDLLA)[28].

Generally, the size of spherical block copolymer micelles range in size from 10 nm to 100 nm[29], and, as mentioned earlier, the size of the micelles is directly related to the circulation time and biodistribution. Smaller sizes improve the circulation time as well as decreasing the chance of uptake by RES.

Therefore, those factors determining the size of the polymeric micelles are extremely important for the overall efficiency as a drug delivery system. The length and molecular weight of the core-forming block polymer is a crucial factor that not only determines the size of the micelles but also plays a role in achieving efficient encapsulation of the hydrophobic drug molecules.

The term "loading efficiency" has been used to determine the amount of the drug that can be incorporated into the core of polymeric micelles. There are several factors that affect loading efficiency, which include total molecular weight and concentration of the copolymer, the nature and concentration of the solute (drug), the length and nature of the corona forming block, and the method of preparation of the delivery system[22].Depending upon the ratio of hydrophilic to hydrophobic

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The type of structure formed is related to the inherent curvature of the molecule, which can be estimated through calculation of its dimensionless packing parameter, *p*.

Materials based on poly (ethylene glycol) (PEG), poly (lactic acid) (PLA), poly (glycolic acid) (PGA) have been widely used in various biomedical applications owing to their biocompatibility and biodegradability. The syntheses of block copolymers derived from these materials have been extensively explored for use as drug delivery systems[31, 32]. Depending on the molecular weight of the blocks, PEG-PLA copolymer can form micelles in aqueous solutions because of their amphiphilic nature. In addition, they can also be used to produce nanoparticles with solid cores, enabling them to encapsulate drugs with widely differing solubility properties. The "stealth" properties of PEG[33,34], which have been utilised to enhance pharmaceutical profiles (e.g. prolong circulation time) of therapeutics ranging from small molecules to proteins, can be combined with controllable biodegradability of PLA/PGA co-polymers (PLGA) to produce highly effective carriers for a variety of drug compounds[35].

Conventionally, diblock copolymers based on biodegrable and biocompatible materials such as poly (L-lactic acid) (PLA), poly (DL-lactic-co-glycolic acid) and poly(ε-caprolactone) (PCL) have been prepared by ring opening polymerisation using a hydrophilic PEG segment as macro-initiator to produce an A-B type di-block copolymer structure.

Hyuk Sang Yoo *et al*, described the synthesis of diblock copolymer composed of poly(DL-lactic-co-glycolic acid) (PLGA) and polyethylene glycol (PEG) which self assemble in aqueous media to form a micellar structure.

The diblock copolymer PLGA-PEG was prepared by Ring opening polymerisation (ROP) using monofunctional PEG (using a fixed molecular weight 2kDa) and tin (II) (2-ethylhexanoate) as macro-initiator and catalyst, respectively. The hydrophobic drug doxorubicin (DOX) (antitumor agent) was encapsulated in the core of the micelles using conjugation technique[36].

Similarly, Xintao Shuai *et al* explored the synthesis of diblock copolymers of poly (ε-caprolactone) (PCL) and monomethoxy poly (ethylene glycol) (MPEG) with various compositions. They have shown that the amphiphilic block copolymers PCL-PEG self-assembled into nanoscopic micelles and their hydrophobic cores used to encapsulate doxorubicin (DOX). In the same manner, the diblock copolymers PCL-PEG with various compositions were prepared by ROP in which the MPEG (fixed molecular weight) was used as macro-initiator in the presence of tin (II) (2-ethylhexanoate) as catalyst[37].

However, optimisations of the carrier system based on PEG and PLA/PGA co-polymers to fine tune the pharmaceutical carriers are difficult and limited to the availability of PEGs with appropriate functional chain ends and ranges of molar mass. In addition, functionalisation of the PEG-PLA/PGA carriers with monofunctional PEG or heterobifuntional PEG is difficult and very costly.

Alternatively, PEG -methacrylate (PEGMA), can be synthesised via a variety of routes, particularly via living radical polymerisation techniques which allow for fine manipulation of the properties through controlling the molecular weight of the block.

Since the discovery of living free radical polymerisation methods, block copolymers are now more easily accessible with control over the molecular weight of the block copolymer and the ratio between the blocks. This can be obtained with narrow molecular weight distribution. The main and widely-used types of living free radical polymerisation techniques are atom transfer radical polymerisation (ATRP) and reversible addition fragmentation transfer (RAFT) [38-42].

The usage of controlled radical polymerisations, primarily ATRP[43-45] (Atom Transfer Radical Polymerisation) and RAFT[46, 47] have enabled PEG-methacrylate and co-polymers with exciting new properties to be produced[48, 49]. Here is shown a combination of ring-opening polymerisation with RAFT agent initiated growth of PEG-methacrylate to yield co-polymer micelles with controllable properties, and with promise as drug-encapsulation and release systems.

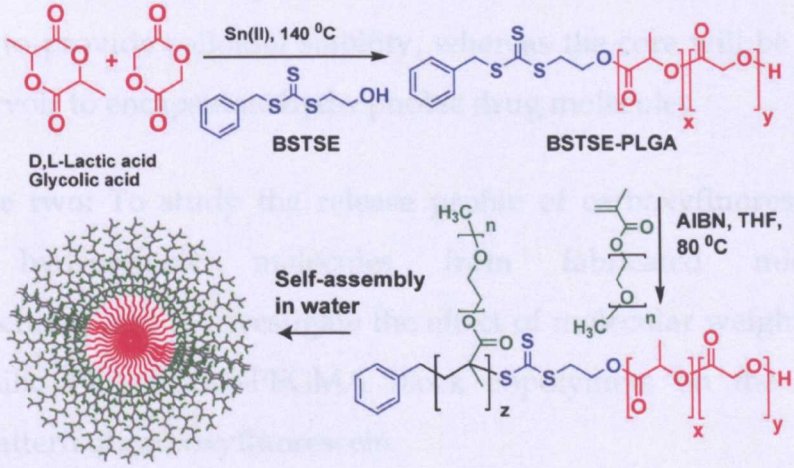


Figure 3-2. Synthesis of PLGA polymer, PLGA-PEGMA block copolymer and assembly into micelles.

3.1. Aims and Objectives

The primary aim of this chapter was to utilise the advanced controlled/"living" polymerisation techniques to synthesize well-defined block copolymers to be used in fabrication of core-shell type nanostructures as drug delivery modalities.

Objectives and Hypotheses:

Objective one: To synthesize a series of biocompatible PLA/PLGA-PEGMA block copolymers, combining ring opening polymerisation (ROP) with Radical Addition Fragmentation Transfer (RAFT) techniques. And also, preparation of micellular type nanostructures, using the synthesised block copolymers.

Hypothesis: Amphiphilic block copolymers based on PLA/PLGA-PEGMA can self assemble in aqueous medium to form core-shell type nanostructures. The hydrophilic chains will be extended in the aqueous medium to provide colloidal stability, whereas the core will be served as a reservoir to encapsulate hydrophobic drug molecules.

Objective two: To study the release profile of carboxyfluorescein as chosen hydrophobic molecules from fabricated micellular nanostructures, and to investigate the effect of molecular weight of the amphiphilic PLA/PLGA-PEGMA block copolymers on the release profile pattern of carboxyfluorescein.

Hypothesis: Hydrolytic degradation of PLA/PLGA blocks in the micellular nanostructure allows the release of carboxyfluorescein from the micelles in a controlled release manner. The release profile of carboxyfluorescein can be fine tuned by changing the molecular weight of the copolymers as well as the diameter size.

3.2. Materials and Methods

3.2.1. Instrumentation

NMR (^1H and ^{13}C) spectra were recorded on a Bruker 400 spectrometer at 399.8 MHz (1H) and 100.5 MHz (^{13}C) using $CDCl_3$ as solvent. Molecular weights and molecular weight distributions were determined using a Varian/Polymer Laboratories GPC-50 instrument with triple detection (RI, viscometry and MALLS). The samples were chromatographed using chloroform as solvent with a flow rate of 1 mL min^{-1} and the molecular weights were calibrated to polystyrene standards. Dynamic light scattering (DLS) data were obtained on Malvern Zetasizer 2000 and Viscotek DLS instruments. Particle size distributions via DLS were derived from correlation functions obtained using OmniSIZE 2.0 software. Diffusion coefficients and hydrodynamic radii were calculated using the Stokes Einstein equation assuming particles were spherical and non-interacting.

Distributions shown are based on particle masses. Fluorescence spectra were recorded using a Varian Cary Eclipse fluorescence spectrophotometer equipped with a Peltier apparatus for temperature control.

DL- Lactide and glycolide were obtained from Purac biochem. The monomers were recrystallised from ethyl acetate prior to use. Poly (ethylene glycol) methyl ether methacrylate (Mw 475) and tin (II) 2-ethylhexanoate were purchased from Sigma. The radical initiator, N, N-azobis (isobutyronitrile) (AIBN, 98%, Aldrich) was recrystallised from ethanol. Solvents were all of HPLC grades. All other reagents were purchased from Fisher Scientific or Aldrich and used as received.

3.2.1. Polymer syntheses

3.2.1.1 Synthesis of BSTSE (RAFT agent) (1)

Synthesis of 2-(Benzylsulfanylthiocarbonylsulfanyl) ethanol (BSTSE). To a solution of potassium hydroxide (13 g, 250 mL, 0.23 moles) was added 20 mL of 2-mercaptoethanol (0.23 moles). 30 mL of carbon disulfide was then added dropwise and the orange solution was stirred for 5 hours. The mixture was then heated to 80 °C and 39.6 g benzyl bromide (0.23 moles) was added. After reaction for a further 12 hours, the mixture was cooled and the aqueous phase extracted with chloroform (3 x 200 mL). The organic layers were then dried over anhydrous magnesium sulphate, filtered and the solvent evaporated. The product was purified by passing through a silica column with hexane/ethyl acetate as eluent (7:3) to yield a yellow liquid (70% yield).

3.2.1.2. Synthesis of BSTSE-PLA polymer (P 1)

PLA, Poly (DL-lactide) was synthesised by ROP (ring opening polymerisation) using BSTSE as initiator. First, DL-lactide (2.8g, 0.02 moles), and BSTSE (0.15g, 0.0005 moles) were added to a polymerisation tube and purged with argon several times before being heated in an oil bath to 80 °C and purged with argon for a further two hours. The reaction mixture was then heated to 140 °C in order to conduct the polymerisation in the melt phase. At this point, tin 2-ethylhexanoate (0.022g, 0.00005 moles) was added, and the mixture was stirred for 24 hours. The polymerisation tube was then removed from the oil bath and cooled down to room temperature. The polymer was recovered by dissolution in THF and precipitated in methanol (this was repeated three times to remove unreacted monomer). The precipitate was filtered and dried under reduced pressure to yield a brown-yellow product (yield 90%). The molecular weight of the polymer and

polydispersity index was determined by gel permeation chromatography using chloroform as eluent.

3.2.1.3. Synthesis of BSTSE-PLGA polymer (P 2)

PLGA, Poly (DL-lactide-co-glycolide) was synthesised by an exactly analogous route to synthesis of P1. First, DL-lactide (1.6g, 0.01 moles), glycolide (1.3g, 0.01 moles), and BSTSE (0.3g, 0.001 moles) were added to a polymerisation tube and purged with argon several times before being heated in an oil bath to 80 °C and purged with argon for a further two hours. The reaction mixture was then heated to 140 °C in order to conduct the polymerisation in the melt phase. At this point, tin (II) (2-ethylhexanoate) (0.049g, 0.0001 moles) was added, and the mixture was stirred for 24 hours. The polymerisation tube was then removed from the oil bath and cooled down to room temperature. The polymer was recovered by dissolution in THF and precipitated in methanol (this was repeated three times to remove unreacted monomer). The precipitate was filtered and dried under reduced pressure to yield a brown-yellow product (yield 95%). The molecular weight of the polymer and polydispersity index was determined by gel permeation chromatography using chloroform as eluent.

3.2.1.4. Synthesis of PLA-PEGMA copolymer (P 5)

The PLA-PEGMA block copolymers were prepared by free radical polymerisation mediated by the macro-RAFT agent (BSTSE-PLA). BSTSE-PLA (0.66g, 0.00012 moles), Poly (ethylene glycol) methyl ether methacrylate (Mn 475 1.46g, 0.003 moles) and AIBN (0.004g, 0.00002 moles) were added into a round bottom flask, the flask purged with argon several times, followed by addition of anhydrous THF (5ml) under an argon atmosphere.

The polymerisation mixture was stirred until the components were completely dissolved. The mixture was then heated in an oil bath to 80 °C and stirred for 24 hours. The polymerisation product was dissolved in THF and reprecipitated three times in methanol, and the filtrate was dried under reduced pressure (yield 85%).
The product was characterised by ^{1}H-NMR. GPC with chloroform as the eluent was used to determine the molecular weight and polydispersity of the final block copolymers.

3.2.1.5. Synthesis of PLGA-PEGMA copolymer (P 6)

The PLGA-PEGMA block copolymers were prepared by exactly analogous route to P5. BSTSE-PLGA (0.66g, 0.00012 moles), Poly (ethylene glycol) methyl ether methacrylate (Mn 475 1.44g, 0.003 moles) and AIBN (0.004g, 0.00002 moles) were added into a round bottom flask, the flask purged with argon several times, followed by addition of anhydrous THF (5ml) under an argon atmosphere. The polymerisation mixture was stirred until the components were completely dissolved. The mixture was then heated in an oil bath to 80 °C and stirred for 24 hours. The polymerisation product was dissolved in THF and reprecipitated three times in methanol, and the filtrate was dried under reduced pressure (yield 85%). The product was characterised by ^{1}H-NMR. GPC with chloroform as the eluent was used to calculate the molecular weight and polydispersity of the final block copolymers.

3.2.1.6. Preparation of Blank PLGA-PEGMA micelles

The PLA and PLGA-PEGMA polymeric micelles were prepared as follows: copolymer (50mg) was dissolved in acetone (3ml), and then distilled water (10ml) was added to the solution under stirring. Mixing was continued in order to facilitate evaporation of the acetone.

Residual acetone was then completely removed by rotary evaporation under reduced pressure. The remaining aqueous solution was slightly cloudy, confirming the formation of micelles. The final micelle concentration was 5mg.mL^{-1}. The mean hydrodynamic radii of the polymeric micelles were determined by dynamic light scattering (DLS).

3.2.1.7. Determination of critical micelle concentration (CMC) of PLGA-PGMA micelle

The CMC of PLGA-PEGMA copolymer micelles was determined using pyrene as a hydrophobic fluorescent probe, as previously described.[50] Briefly, an aliquot of pyrene solution (6×10^{-6} M in acetone, 1mL) was added to 6 vials, and the acetone evaporated at 60 °C. The aqueous solutions containing copolymer of various concentrations were then added (10 mL) to the vials containing pyrene. The final concentration of pyrene in each vials was 6×10^{-7} M. The solutions were kept at room temperature for 24 h to reach the pyrene solubility equilibrium in the aqueous phase. Emission was carried out at 390 nm, and excitation spectra were recorded over the range 240 - 360 nm. From the pyrene excitation spectra, the intensities at 337 nm were analysed as a function of the polymer concentrations. The CMC value was determined from the intersection of the two curves.

3.2.1.8. Dynamic light scattering

The PLA and PLGA-PEGMA polymeric micelles (P5, P8) were prepared as mentioned before. The micelles solution was filtered with a 1 μm pore-size filter to eliminate dust contamination. A 60 μL aliquot was added in a quartz cuvette and the sample was examined on a Viscotek 802 dynamic light scattering instrument. Scattered light coming from a 50 mW laser source (830 nm) was recorded from an internal light detector aligned at 90° from source. From standard auto correlation functions, measured correlation coefficients were related to hydrodynamic radii at varying temperatures via the Stokes-Einstein equation, $R_H = K_bT/6\pi\eta D$, where R_H is the hydrodynamic radius, k_b is the Boltzmann constant, T is the temperature and η is the viscosity of the solvent. The size distribution of the scattering particles was determined by the average mass number of the particles.

3.2.1.9. Microscopy

Scanning Electron Microscope samples were prepared by placing a droplet of an aqueous micelles solution (**P5, P8**) on a carbon grid and left to dry for a few minutes at room conditions. Standard plasma coating with gold was performed for two minutes before sample examination.

3.3. Release time profile of carboxyfluorescein from PLGA-PEGMA micelles

PLGA-PEGMA copolymer (200 mg) and carboxyfluorescein (20 mg) were co-dissolved in acetone (3 ml). The solution was then added dropwise into 10mL distilled water under stirring. The acetone and part of the water were then evaporated under reduced pressure.

The micellar solutions were passed through a Sephadex column (PD-10 Desalting column) to remove excess of un-entrapped

carboxyfluorescein. The micelle fractions were subsequently put into 500mL distilled water to study the release of the dye over time. Samples were collected from the media at pre-determined intervals (correction for volume changes due to sampling were made) and emission spectra were recorded from 502 nm to 600 nm at excitation wavelength of 492 nm using fluorescent spectrophotometer

3.4. Cellular uptake of PLGA-PEGMA micelles by 3T3 fibroblast cell line

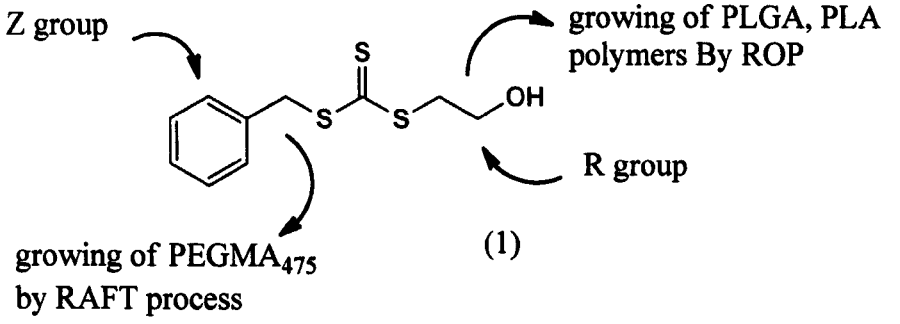
3T3 mouse fibroblasts were seeded at a density of 10,000 cells.mL^{-1} in 24-well tissue culture plastic plates and allowed to attach for 24 hrs in DMEM supplemented with L-glutamine (2mM), antibiotic/antimycotics (penicillin-100units.mL^{-1}, streptomycin-0.1mg.mL^{-1}, amphotericin B-0.25µg.mL^{-1}), foetal calf serum (FCS) (10%). The cells were then washed with warm PBS and incubated with a suspension (100ul) of FITC-loaded PLGA-PEGMA micelles (0.2 mg. mL^{-1}) in HBSS (Hank's Balanced Salt Solution) for 0 min, 15 min, 1hr, 2hr and 6 hrs at 37 °C. Cell monolayers were subsequently washed with PBS several times prior to microscopy. For nuclei staining, DAPI standard solution (Invitrogen, 1 drop) was added to each well and the stain allowed to enter the cells over a period of 10 min. Cells were imaged using a Leica DM IRB microscope and images were processed using Leica DC200 using DC viewer, version 3.2.0.0 software.

3.5. Results and Discussion

3.5.1. Polymer syntheses

In the past decade, poly (lactide) PLA and poly (D, L- Lactide-co-glycolic) PLGA polymers have been used in various biomedical applications[51]. Recently, ring opening polymerisations of these materials have gained increasing interest because of growing demand for biocompatible and biodegradable materials. As it has been mentioned in (chapter 1 Section 1.7.1), ring opening polymerisation can be conducted by a numbers of metal complexes, however, tin (II) 2-ethylhexanoate remain the favoured catalyst even for industrial production as it has approval from the Food and Drug Administration (FDA). Therefore we have chosen to use tin (II) 2-ethylhexanoate to catalyse the polymerization of DL, lactide and glycolide monomer.

It has been reported that, in order to obtain PLGA based polymer with control over the molecular weight and in relatively fast polymerisation rate, the tin (II) 2-ethylhexanoate catalyst has to be combined with a protic agent, typically an alcohol. The aim of this work was to synthesize the diblock copolymer composed of PLGA and $PEGMA_{475}$. The latter is considered hydrophilic and also $PEGMA_{475}$ has been successfully polymerised by RAFT process with good control over the molecular weight and polydispersities. Therefore, to prepare PLGA-$PEGMA_{475}$ diblock copolymer, first, BSTSE (2-benzylsulfonylthiocarbonylsulfonyl) ethanol was used as a heterobifuntional RAFT agent. BSTSE has one hydroxyl group (to use as a protic agent) on the z-group of the trithiocarbonate chain-transfer agent to initiate the ring opening polymerisation of lactide and glycolide monomers in the presence of tin (II) 2-ethylhexanoate catalyst.



Structure of BSTSE (RAFT agent)

Using lactide monomers alone or with glycolide monomer at the ratio of 50:50 resulted in both PLA and PLGA polymers which were end-capped with BSTSE as the ring opening polymerisation propagated from the hydroxyl group of the BSTSE agent. The ratio of 50:50 of lactide to glycolide monomer was chosen as this is known to give polymers with a faster degradation rate compared to other ratio of 75:25 of 80:20

BSTSE agent and tin (II) 2-ethylhexanoate rapidly generated a complex-equilibrium forming highly reactive alkoxides from the alcohol. Varying the concentration of BSTSE agent compared to the concentration of lactide and glycolide monomers, it was possible to prepare different molecular weight of PLGA-end capped with BSTSE and relative good control over the molecular weight distribution (see Table 3-1, P1 - P4)

The PLA and PLGA-end capped with BSTSE was isolated by dilution in THF and subsequent precipitation in methanol. It should be noted that Methanol is an excellent solvent for the low molecular weight BSTSE (RAFT agent) (see the structure of BSTSE in Figure 3-2, compound 1) and lactide and glycolide monomers. So residual BSTSE and unreacted monomers can be efficiently removed by using this purification method.

The RAFT process has been shown to provide an extremely versatile route to well-defined macromolecular architectures and polymers or copolymers exhibiting narrow polydispersities[52, 53]. PEG-methacrylate monomers have been successfully polymerised using RAFT process with very good control of the molecular weight distribution.

In this work, $PEGMA_{475}$ monomers were polymerised using PLA, PLGA-end capped with BSTSE agent.

As a result of this polymerisation a series of amphiphilic block copolymers were prepared, which are composed of a highly hydrophilic segment of Poly(ethylene glycol) methyl ether methacrylate as one block and hydrophobic segment of Poly (lactic acid) or Poly(D,L-lactic acid-co-glycolic acid) as second block following the concept of combining two different types of polymerisation, ROP and RAFT.

Good control over the molecular weight dispersity was achieved and good agreement between the experimental and theoretical molecular weight was observed (P5-P8, Table 3-I). It worth noticing that the polydispersity of the block copolymers P5 - P8 after the RAFT process was similar to that of the precursor polymers P1 - P4, indicating a good level of control in the RAFT-mediated reactions.

These block copolymers thus combined the properties of being biodegradable and biocompatible, and have well-defined structure with controlled molecular weight and polydispersity. Although poly(caprolactone) and PEG-methacrylate polymers have very recently been described,[54] perhaps surprisingly there were no prior reports of PLGA-$PEGMA_{475}$ block co-polymers synthesised by controlled routes at the start of this study.

The chemical syntheses of the polymers used in this study are shown in Figures 3-3 and 3-4.

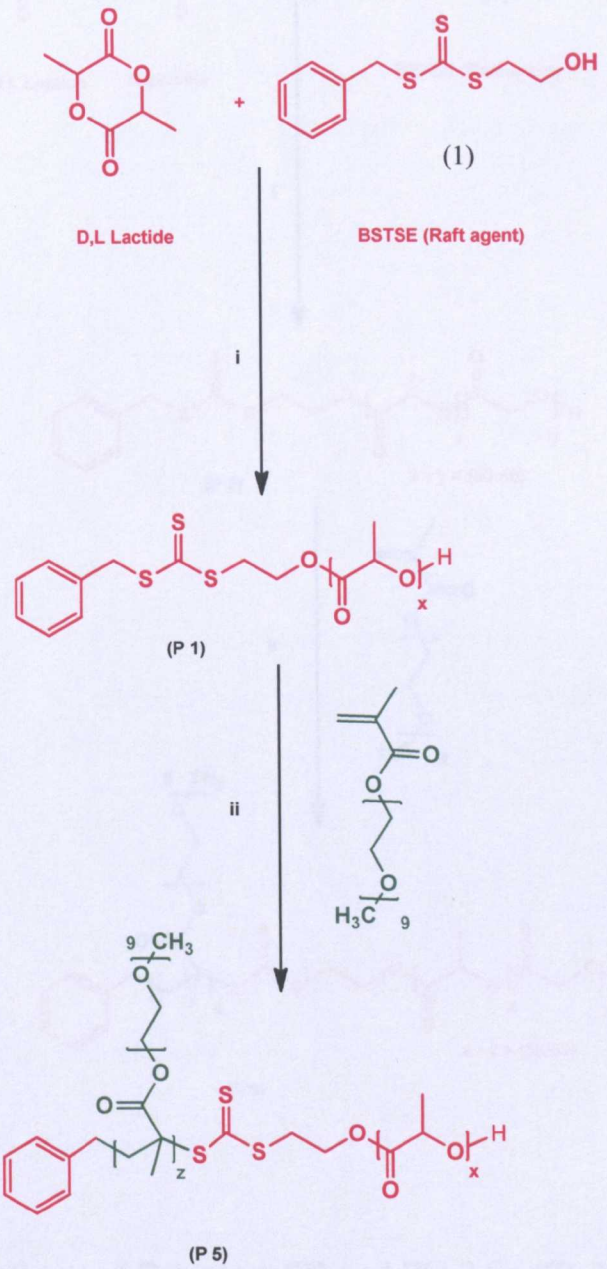


Figure 3-3. Syntheses of Polymers (P1 and P5) i) Sn (II), 140 oC, 24 h, ii) THF, AIBN, 80 °C 24 h, (see Materials and Methods section).

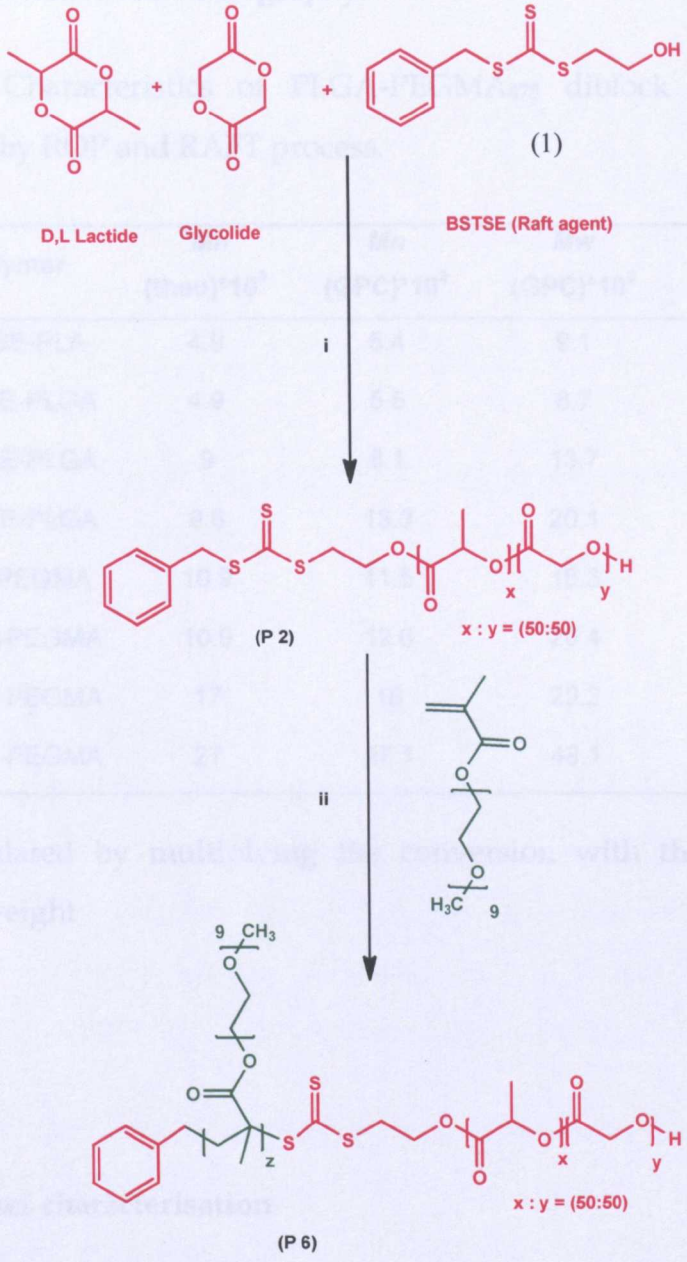


Figure 3-4. Synthesis of Polymers (P2 and P6) i) Sn (II), 140 oC, 24 h, ii) THF, AIBN, 80 °C 24 h, (see Materials and Methods section).

The properties of PLA / PLGA -$PEGMA_{475}$ copolymers synthesised by ROP and RAFT are summarised in table 3-1, describing the molecular

weight and molecular weight distributions based on results obtained from gel permeation chromatography.

Table 3-I. Characteristics of PLGA-PEGMA$_{475}$ diblock copolymer synthesised by ROP and RAFT process.

Entry	Polymer	[a]Mn (theo)*10^3	Mn (GPC)*10^3	Mw (GPC)*10^3	Mw/Mn
P 1	BSTSE-PLA	4.9	5.4	9.1	1.6
P 2	BSTSE-PLGA	4.9	5.5	8.7	1.5
P 3	BSTSE-PLGA	9	8.1	13.7	1.5
P 4	BSTSE-PLGA	9.6	13.3	20.1	1.5
P 5	PLA-PEGMA	10.9	11.5	16.3	1.4
P 6	PLGA-PEGMA	10.9	12.6	20.4	1.6
P 7	PLGA-PEGMA	17	18	29.2	1.6
P 8	PLGA-PEGMA	27	27.1	48.1	1.7

[a] was calculated by multiplying the conversion with the targeting molecular weight

3.5.2. Polymer characterisation

3.5.2.1 NMR spectroscopy and FTIR

The ^{1}HNMR spectrum of BSTSE compound was studied in ($CDCl_3$) (Figure 3-5.); this compound has been prepared as mentioned in literature. The structure of the compound was confirmed by comparison with literature data. It was possible to assign the peaks from the spectrum of BSTSE as follow: the peak of 3.59 (t, 2H, CH_2-O), 3.85 (t, 2H, CH_2-S), 4.62 (s, 2H, CH_2-Ph), 7.31 (m, 5H, Ph). FTIR (cm^{-1}) 2860–2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

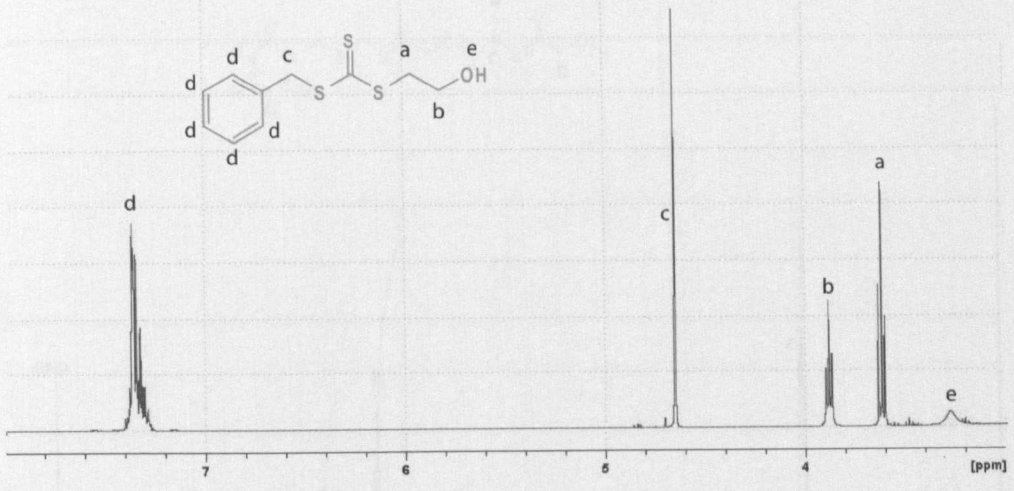


Figure 3-5. ^{1}HNMR spectrum of BSTSE (RAFT agent) (representative of trithiocarbonate RAFT agent).

The structure of the PLGA polymer end-capped with BSTSE was also confirmed from ^{1}H NMR spectrum obtained in ($CDCl_3$) (Figure 3-6.). The peaks which are labelled in Figure 3-6 referred to the protons of the backbone of PLGA polymer (a-c) and the end group for BSTSE (d). δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H,CH_2- C=O), 1.58 (d, 3H, CH_3-CH), 7.3-7.4 (m, 5H, Ph). FTIR (cm^{-1}) 2860–2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

Owing to the low intensity of the phenyl proton adsorption compared to the protons of the PLGA in the ^{1}H NMR spectrum, it was difficult to calculate the molecular weight via ^{1}H-NMR alone, however using the integration on the spectrum, it was possible to confirm the 50:50 ratio of lactide to glycolide units in the polymer which was the target in the synthesis of the polymer.

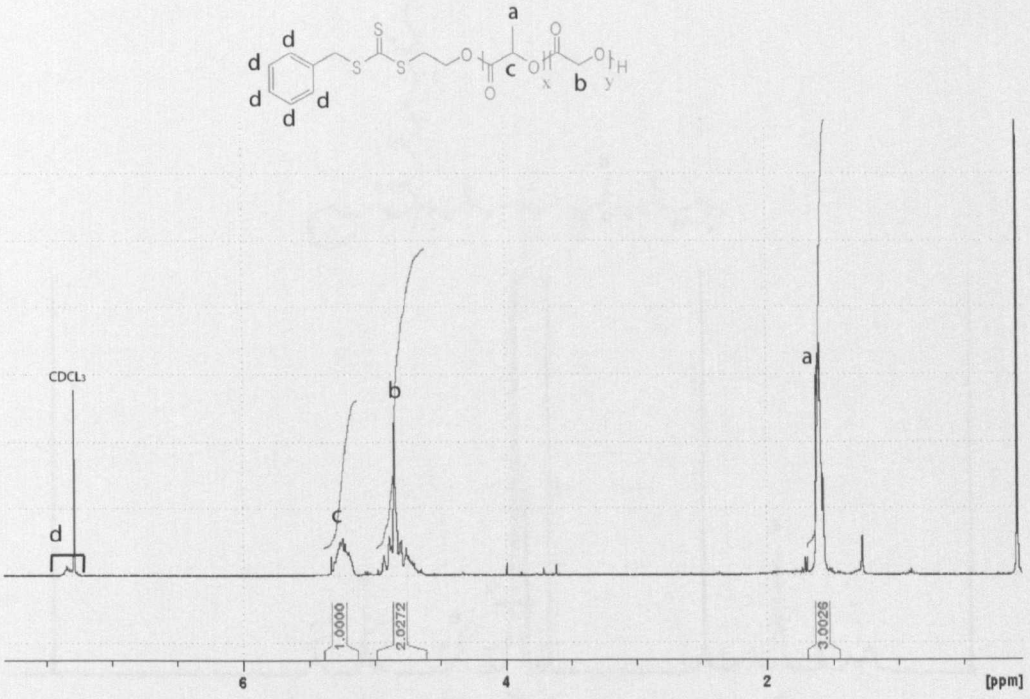


Figure 3-6. ^{1}HNMR spectrum of BSTSE-PLGA

From the ^{1}H NMR spectrum of PLGA-PEGMA$_{475}$ block copolymer, it was possible to characterise the structure by assigning the peaks as shown in (Figure 3-7). The peaks labelled from e-g were assigned to the protons on the PLGA block. The peaks labelled (h) were assigned to PEGMA units.

δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H, CH_2- C=O), 3.94-4.14 (m, CH_2, CH_2-O- C=O), 3.4-3.8 (m, CH_2CH_2O), 1.58 (s, 3H, CH3), 1.6-1.9(m, CH_2), 0.7-1.3 (s, CH3). FTIR (cm^{-1}) 2860–2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

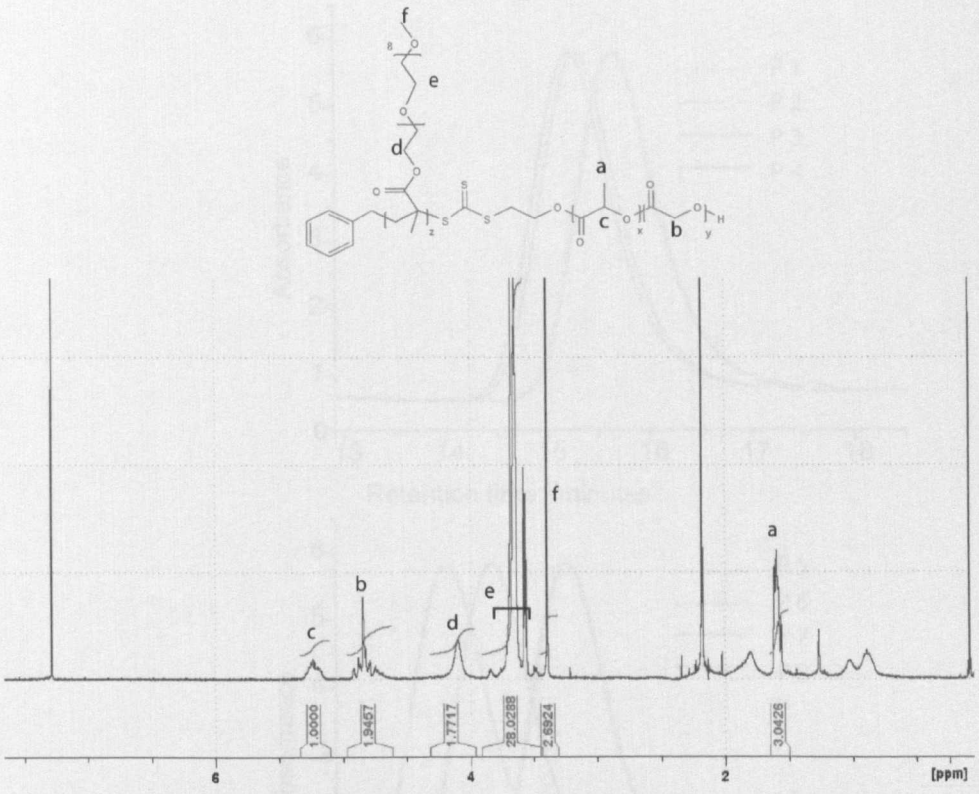


Figure 3-7. 1HNMR spectrum of PLGA-PEGMA.

3.5.3. Molecular weight determination

Gel permeation chromatography was used to determine the molecular weight and polydispersity of the polymers. The GPC traces of BSTSE-terminated PLGA polymers (P 1-P 4) and PLA/PLGA block copolymers (P 5-P 8) are shown in Figure 3-8.

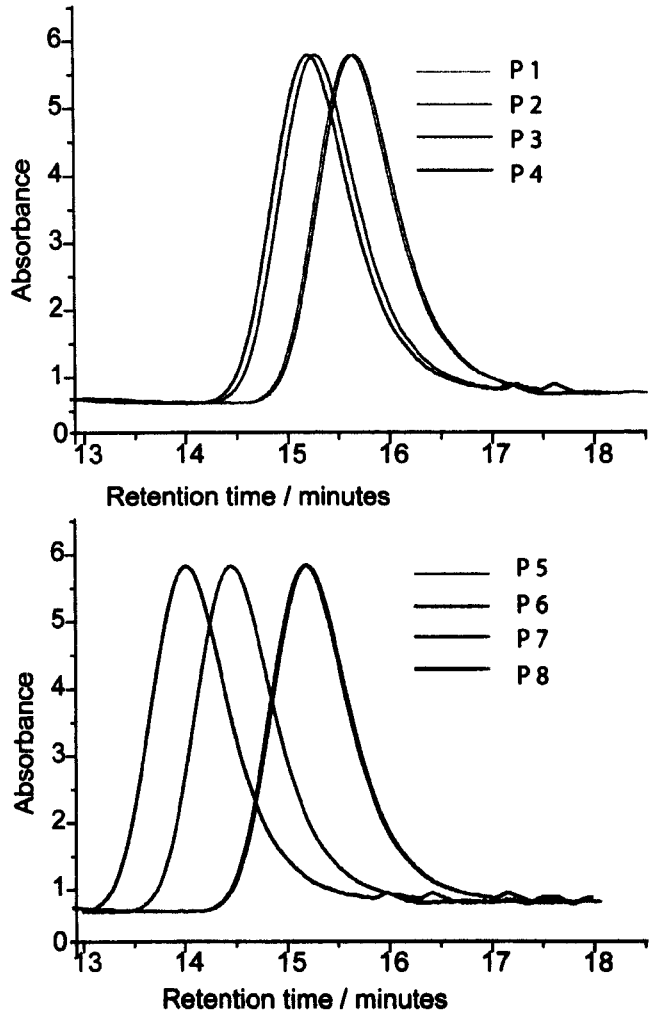


Figure 3-8. GPC chromatographs for polymers (see Table 3-I) in chloroform eluent. Note, some traces are overlapped due to similarity in retention times.

The molecular weights and the polydispersity indices of the polymers BSTSE-PLGA polymers and PLGA-PEGMA$_{475}$ diblock copolymer were determined by GPC (Gel permeation chromatography), and summarised in Table 3-1. The PDIs of BSTSE-PLGA polymers (P 1-P 4) are between 1.5-1.6 which is typical for polymers produced by ring opening polymerisation.

On the other hand, the PDIs of PLGA-PEGMA$_{475}$ diblock copolymers (P5-P8) are slightly different from the BSTSE-PLGA polymers which indicate the efficiency of RAFT polymerisation. The GPC results showed a decrease in retention time of PLGA-PEGMA in comparison to the GPC curve of the first block (BSTSE-PLGA). This was a preliminary indication that the second block (PEGMA) was inserted to the polymer chain between the trithiocarbonate unit and PLGA.

The curves were unimodal and symmetric indicating relatively good control over the polymerisation. This is typical for tin (II) catalysed ROP of the monomers using standard hydroxyl initiators. The GPC curves for the PLGA-PEGMA$_{475}$ (P5-P8) block copolymers (see Figure 3-8) indicated progressive chain extension of the PLGA upon block copolymerisation with PEGMA$_{475}$.

There was a slight change in the molecular distribution of PLGA-PEGMA in relative to BSTSE-PLGA which confirmed the efficient RAFT process. More importantly, all GPC traces were unimodal suggesting that block copolymers were indeed formed and that the presence of either PLGA or PEGMA homopolymer was low.

3.5.4. Micelle formation and characterisation

The amphiphilic nature of the block copolymer PLGA-PEGMA provided the opportunity to form water stable micelles. The hydrophilic PEGMA segment was expected to form the shell of the micelle structure whereas the PLGA segments assembled into a hydrophobic core. Pyrene fluorescence emission intensity spectra were used to evaluate critical micelle concentrations (CMC) owing to the sharp increase in fluorescence intensity as the hydrophobic dye transferred into the micellar cores. CMC values obtained from these plots were in the range 0.3- 1mg.L^{-1} (Figure 3-9).

Size distributions of co-polymer micelles were determined by dynamic light scattering above the CMC. The micelles from co-polymers P5-P8 exhibited two populations, with the majority fractions in terms of mass distributions exhibiting radii of ~ 12-16 nm, with a second population consisting of much larger species with diameter 60-180 nm. In the absence of an acid-catalyst, the micelles were stable in PBS, as shown by DLS, over a 24 hr period; this suggests that the micelles and vesicles were stable to aggregation, at least over the time periods of the experiments.

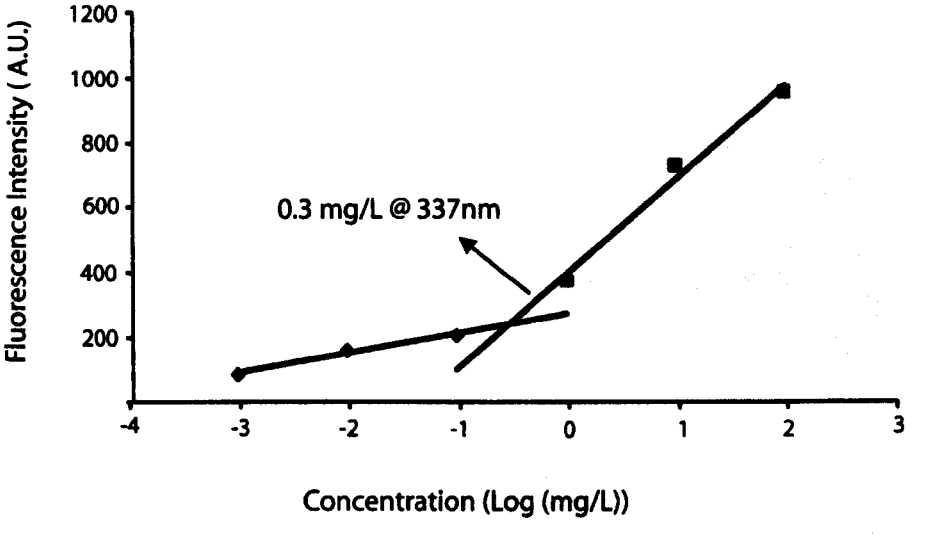


Figure 3-9. Graph representing change in pyrene emission intensity as a function of polymer concentration for PLGA-PEGMA co-polymer 8.

Table 3-II. Dynamic Light Scattering data for polymer micelles

Polymer	Fraction 1		Fraction 2	
	% Area	R_H (nm)	% Area	R_H (nm)
P 5	45.9	15.5	54.1	59.5
P 6	64.1	14.7	35.9	79.5
P 7	71.9	13.6	28.1	128.8
P 8	72.0	11.9	28.0	175.6

Representative DLS plots for Polymers P 5 and P 8 together with TEM images are shown in Figure 3-10.

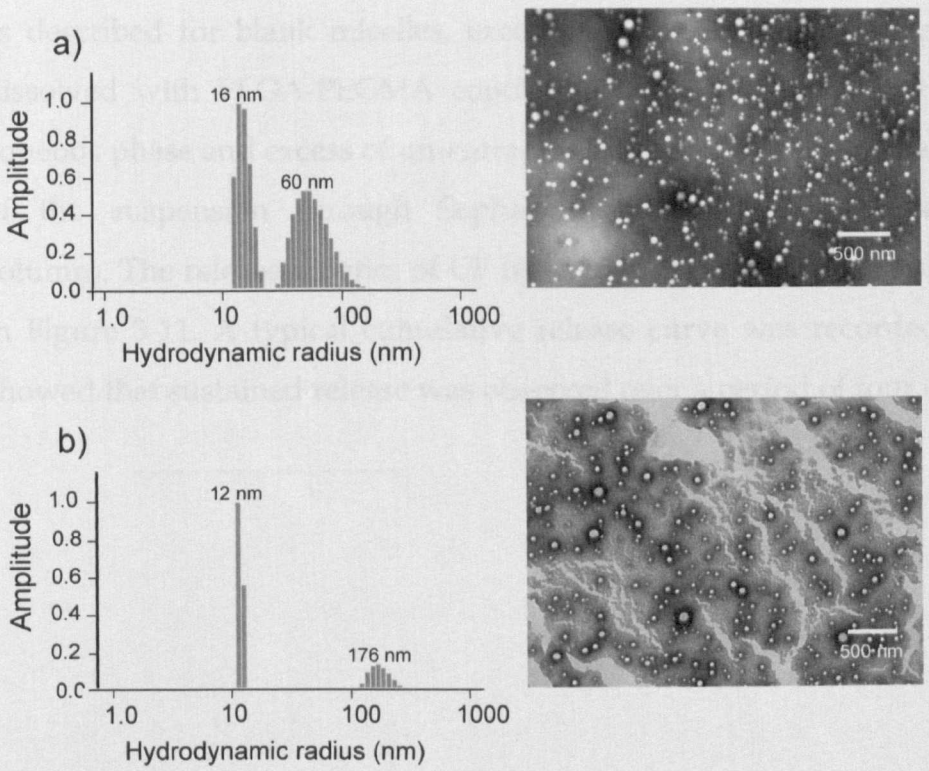


Figure 3-10. DLS and TEM data of block co-polymer micelles formed from polymers P 5 (a) and P 8 (b).

The spherical shape of the micelles is apparent in TEM, and the presence of larger micelle-type assemblies is most clearly seen in the larger DLS components and TEM features in (b) for polymer P 8.

3.5.5. Drug release studies.

Controlled drug release is the longer-term goal of these studies with degradable, hydrophobic core co-polymer micelles. Two dye molecules were chosen as model drugs, carboxyfluorescein (CF) and fluorescein isothiocyanate (FITC), which differ in their hydrophilicity, in order to evaluate drug encapsulation and release by the co-polymers. Carboxyfluorescein is relatively hydrophilic but unreactive, and thus is a good candidate to evaluate incorporation into hydrophobic-core micelles. The protocol used to prepare CF-loaded micelles was the same as described for blank micelles, except that the model drug was co-dissolved with PLGA-PEGMA copolymer prior to addition into the aqueous phase and excess of un-entrapped CF was removed by passage of the suspension through Sephadex columns (PD-10 desalting column). The release kinetics of CF from co-polymers P5-P8 are shown in Figure 3-11. A typical cumulative release curve was recorded and showed that sustained release was observed over a period of four days.

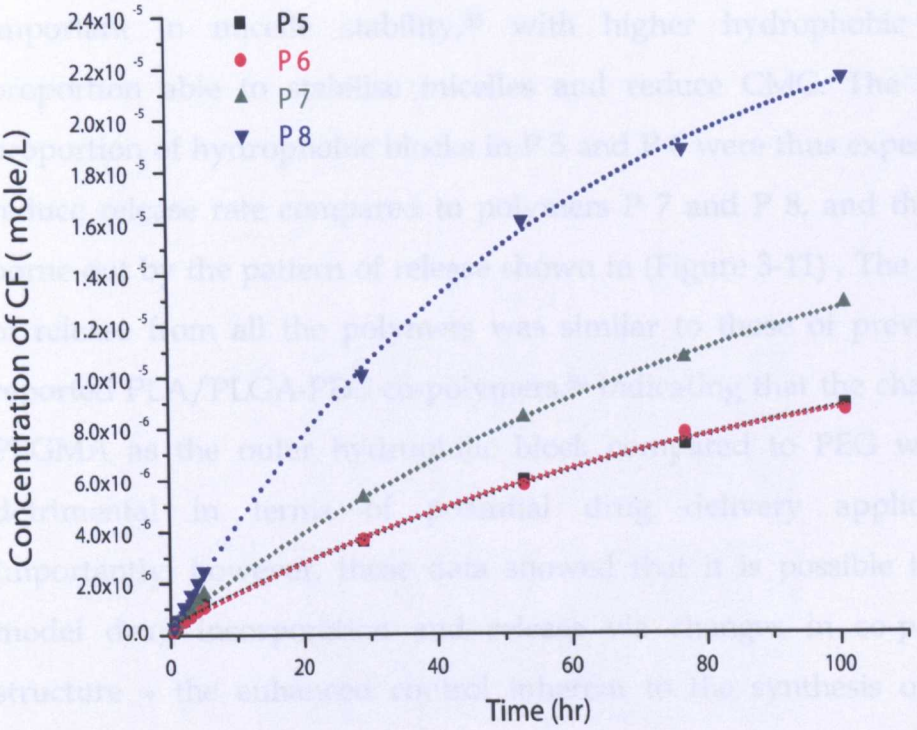


Figure 3-11. Release of carboxyfluorescein (CF) with time from PLGA-PEGMA block co-polymers in water. The starting concentrations of CF and co-polymers used to form the micelles were the same in all cases.

Cumulative release correlated well with PLGA block length, with polymers P 5 and P 6, containing PLGA blocks of Mn 5.5 kDa (GPC) releasing CF at the same rate and to the same extent. By contrast, polymers P 6 and P 7, with Mn (GPC) of PLGA blocks of 8.1 kDa and 13.3 kDa, respectively, released more CF and to a higher overall extent. This also linked with calculated molar volumes by DLS, with P 8 (3.2 x 10^{-2} $\mu m^3.mole^{-1}$) exhibiting several-fold higher cumulative release than P 5 and P 6 (~1.0 x 10^{-3} $\mu m^3.mole^{-1}$).

The relative proportion of hydrophobic to hydrophilic blocks is also important in micelle stability,[55] with higher hydrophobic block proportion able to stabilise micelles and reduce CMC. The higher proportion of hydrophobic blocks in P 5 and P 6 were thus expected to reduce release rate compared to polymers P 7 and P 8, and this was borne out by the pattern of release shown in (Figure 3-11) . The profile of release from all the polymers was similar to those of previously-reported PLA/PLGA-PEG co-polymers,[56] indicating that the change to PEGMA as the outer hydrophilic block compared to PEG was not detrimental in terms of potential drug delivery applications. Importantly, however, these data showed that it is possible to alter model drug incorporation and release via changes in co-polymer structure - the enhanced control inherent to the synthesis of these PLGA-PEGMA block co-polymers by combined ROP and RAFT allows much better flexibility in co-polymer preparation than the anionic routes needed for PLA-PEG systems.

3.5.6. Cell uptake studies of micelle-entrapped dye

Preliminary cell uptake studies were carried out using the 3T3 fibroblast cell line, as a representative target for micellar drug carrier systems.[57] Fluorescein isothiocyanate (FITC) was used as a convenient probe, owing to its accessible excitation and emission wavelengths and its reactivity to amine-containing biomolecules. FITC was considered as complementary to CF as it is more hydrophobic and therefore a better model for more hydrophobic drugs, but also its reactivity would mean that any release into an environment with amine-containing biomolecules would lead to rapid and permanent staining. FITC was incorporated into polymer P5 and P8 micelles using the same methods as for CF, and as before free drug was removed by passage of micelles down a Sephadex column. The loaded micelles were added to the 3T3 cells at 37 °C and at concentrations above CMC: again the cells were

immediately washed to remove any free dye in solution. As is apparent from microscopy (Figure 3-12), uptake was rapid and the FITC fluorescence was apparent in microscopy images of cells incubated with either polymer P5 or P8 within 15 min, with intense fluorescence after one hour.

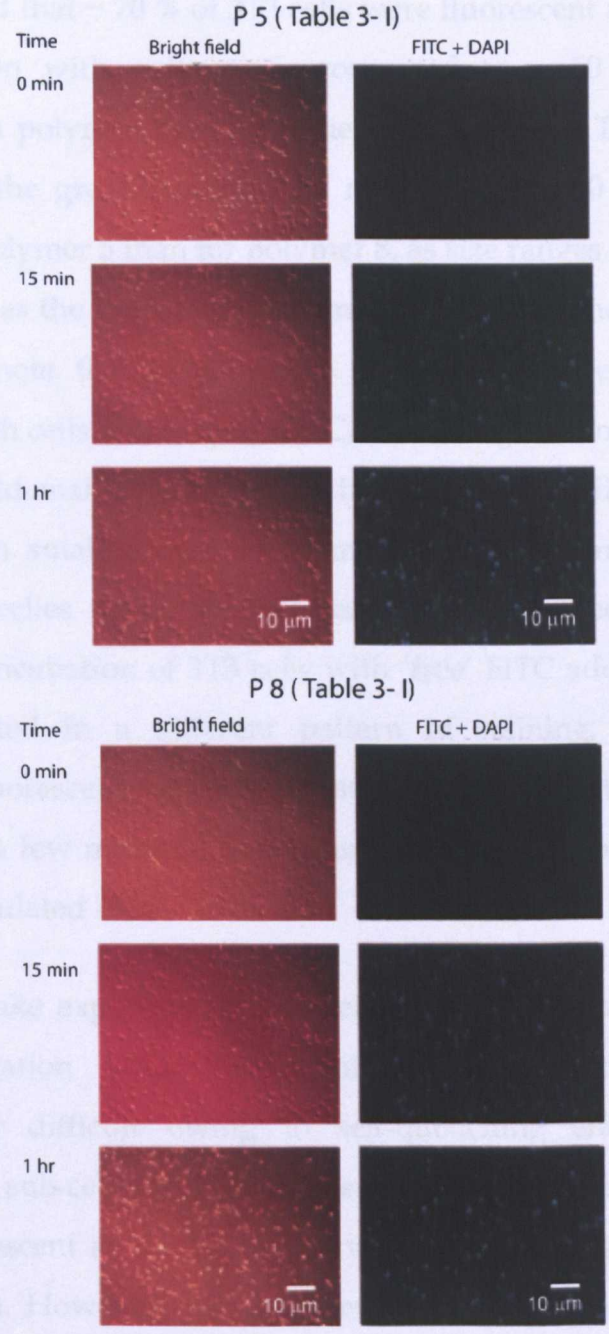


Figure 3.12. Microscopy of 3T3 fibroblasts after incubation with FITC-loaded polymers 5 (top six images) and 8 (bottom six images).

Micrographs obtained in bright field are on the left-side of the image, and combined images obtained under DAPI and FITC filters are shown on the right-hand side.

Analysis of uptake via counts of fluorescent cells detected in FITC and DAPI stains compared to total cell numbers in bright field (Scion Image, NIH) suggested that ~ 70 % of 3T3 cells were fluorescent after one hour after incubation with polymer 5, compared to ~ 50 % for those incubated with polymer 8 at the same concentrations. This was most likely due to the greater number of micelles of < 200 nm diameter exhibited by polymer 5 than for polymer 8, as size ranges of 150-200 nm are often cited as the most highly taken up by passive endocytosis. It is important to note that at all stages the polymers were present in suspension with cells above their CMC, so although the micelle-unimer equilibria would enable some FITC to be liberated, it is likely this effect will have been small compared to micellar uptake and subsequent dilution of micelles inside the cell, leading to enhanced intracellular drug release. Incubation of 3T3 cells with 'free' FITC added to DMEM solution resulted in a different pattern of staining, with a high background fluorescence at t = 0, and strong labelling of the outsides of the cells after a few minutes, in contrast to what was observed for the micelle-encapsulated FITC.

These cell uptake experiments are preliminary and it is also the case that quantification with fluorescent dyes as model drugs is experimentally difficult owing to self-quenching effects and pH differences in sub-cellular compartments (FITC, for example, is not strongly fluorescent at the lower pH values found in late endosomal compartments). However, the data nevertheless shows that these di-block micelles are capable of rapid entry into a representative cell line, an important requirement for any drug delivery system.

3.6. Conclusions

In this chapter it is shown that it is possible to synthesise block co-polymers based on biodegradable PEG-methacrylate and PLGA components in a facile one-pot procedure and with good control over block length, proportion and final molar mass. These copolymers assembled in aqueous solution into micellular nanostructures to encapsulate model drug compound (carboxyfluorescein). Control over the hydrodynamic diameter size as well as the release pattern profile of carboxyfluorescein from the micelles were successfully achieved by choosing copolymers (P5 to P8) which are different in overall molecular weights. Release studies with carboxyfluorescein as a fluorescent model drugs showed that overall release was higher with micelles formed from P8 copolymer compared to P5 and P6, which the latter, showed the lowest releasing profile. Successful uptake of micelles into 3T3 cells was shown for two block co-polymers used, polymers P 5 and P 8.

The combination of ROP and RAFT chemistries[58] used in this chapter to establish a synthetic, flexible route to make PLGA-PEGMA$_{475}$ copolymers offered many advantages compared to existing routes which utilised limited molecular weight PEG based polymers to make PLGA-PEG type copolymers. The tailoring of co-monomer type and the wide variety of methacrylate and acrylate functional materials available that can be polymerised via RAFT or ATRP means that fine-tuning of drug incorporation and release should be possible,[59-62] and therefore, the developed route in this chapter to make such biocompatible copolymers will be very beneficial to the field of controlled drug delivery system.

So far, the successful synthetic platform was developed by utilising advanced polymerisation technique to fabricate biocompatible copolymers to make core-shell type nanostructure. The core-shell type nanostructure, prepared in this chapter, has potentially better colloidal stability compared to physically surface-coated nanostructures which were used in chapter two.

Although the synthesised block copolymers in this chapter can be utilised to make core-shell nanoparticles for delivery of DNA, using conventional double emulsion technique. But, the lack of specific targeting or active release of DNA will hamper their application. Therefore, we have developed another synthetic route that also utilised controlled/"living" polymerisation technique to make the aforementioned block copolymers but also allow the incorporation of bioresponsive functionality (to trigger the release of DNA inside the cell) and to attach targeting moieties. The synthesis of such copolymers to make core-shell nanoparticles is studied in detail in chapter four of this thesis.

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Chapter 4

Multifunctional Bioresponsive Cell-Targeted Block Co-polymer Nanoparticles for Gene Delivery

4. Introduction

Carrier materials for therapeutic biopolymers must fulfil a number of demanding criterias[1],and for nucleic acid delivery the hurdles are especially challenging[2-4]. DNA and RNA delivery vehicles are required to package the polynucleotide, protect it during transport in and across numerous biological environments and barriers, and then release the biopolymer in the correct cellular location. The delivery materials should also be non-toxic, and ideally should be biodegradable. Block co-polymer micelles, vesicles and nanoparticles have been extensively investigated as carriers for nucleic acids[5-8], as the core-shell architectures enable packaging of DNA or RNA into a protected interior phase. In addition, judicious choice of functional monomers, synthetic routes and formulation conditions allows self-assembly into finely-structured nanomaterials that can display bioactive ligands for cell or tissue-specific targeting above a hydrophilic corona for enhanced biodistribution[9]. Prior work[10-16] has established the principle of poly (ethylene glycol) (PEG) block co-polymer micelles for drug and gene

delivery, utilizing the ability of PEG to reduce opsonisation and clearance from the body.

Increased stability via PEGylation can have the downside of reducing cell uptake and accordingly several groups have prepared block co-polymers or conjugates wherein the PEG block is connected to the hydrophobic component or drug via cleavable or reversible/disruptable linkers[17-19]. This enables the PEG-corona to separate from the core, exposing the contents for release, in a manner analogous to the shedding of viral coat proteins. Another strategy for restoring activity of PEGylated or other co-polymer micelles is to introduce cell targeting functionality, for example by attaching a ligand to the outside of the PEG layer that can be recognized by a specific cell surface receptor and marked by the cell for uptake. The overexpression of the folic acid receptor on certain cancer cells has been explored by a number of groups, who have end-functionalised polymer drug delivery systems with folic acid or folates to enhance uptake via folate receptor mediated endocytosis[13, 20, 21].

Unfortunately, the availability and cost of heterobifunctional PEGs for concurrent co-polymer micellisation and ligand decoration has been a limiting factor and so strategies for controlled synthesis of block co-polymers and nanoparticles with more easily functionalisable end-groups have emerged[22,23]. Installation of folate groups at end-groups of polymers prepared by Atom Transfer Radical Polymerization (ATRP) has been carried out with high control and good yields[24].

ATRP offers an additional advantage in that the many commercially available acrylic ester monomers can be polymerized with a high degree of control[25, 26]. A number of poly(ethylene glycol)methacrylate (PEGMAs) can be obtained that vary in side-chain length and water solubility, enabling control not only of molar mass and polydispersity via ATRP, but also the hydrophilicity and architecture of the surface corona of a micelle or vesicle[27-29].

PEGMAs have shown good biocompatibility[30,31] and their combination with a hydrophobic polymer block such as poly(lactic acid-co-glycolic acid) (PLGA - which is widely used clinically), has been shown to produce well-defined and biocompatible nanoparticles[32,33]. For encapsulation of biopolymers such as DNA however, simple micelle formation with these block materials is of limited utility as the nucleic acids do not easily partition to the interior phase. Water/oil/water (W/O/W) emulsion systems can be used to encapsulate nucleic acids via their initially entrapped aqueous regions and block co-polymer surfactants have been used to enhance DNA incorporation into PLGA nanoparticles by this route[34, 35]. PLGA-PEGMA block co-polymers are obvious candidates to use for interfacial stabilization of w/o/w emulsions, while the ability to tune block co-polymer structure in PLGA-PEGMA systems through controlled synthesis also in principle allows for further control of emulsion stability via this route.

In this chapter the combination of multiple factors, i.e. controlled block co-polymer synthesis, functionality for receptor-mediated uptake and the formulation into well-defined nanoparticles via w/o/w /routes leads to selective and active gene delivery vectors. This is achieved through the synthesis of PLGA-S-S-PEGMA diblock and PLGA-S-S-PLGA homo block co-polymers with surface-displayed folic acid functionality for enhanced uptake and DNA release in the clinically-important Calu-3 cell line.

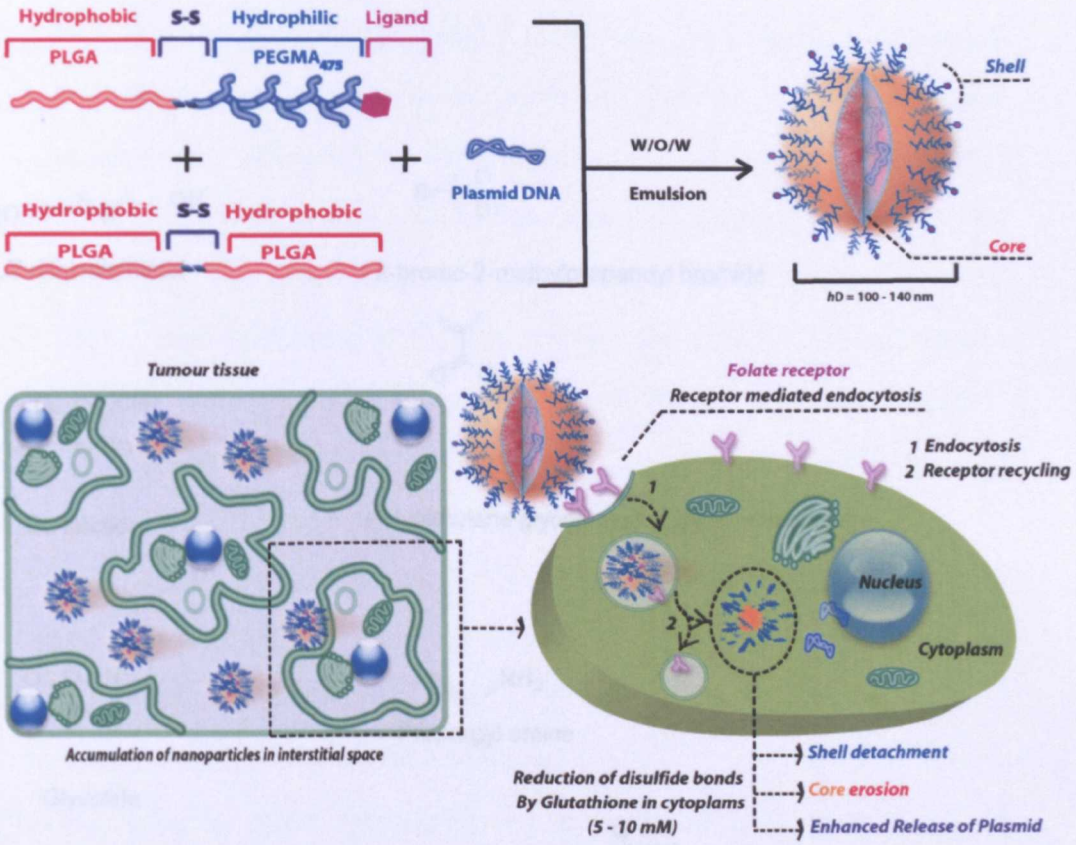
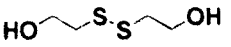
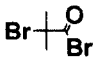


Figure 4-1. Schematic illustration of a) fabrication of folate functionalised nanoparticles; b) accumulation of folate functionalised nanoparticles in interstitial space via EPR; c) folate receptor mediated endocytosis, shell detachment and core erosion of the particles to enhance intracellular release of plasmid DNA.

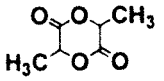
Structure of key compounds used in this chapter



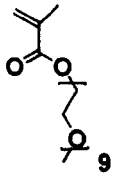
2,2'-dithiodiethanol



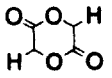
2-bromo-2-methylpropanoyl bromide



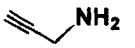
DL-Lactide



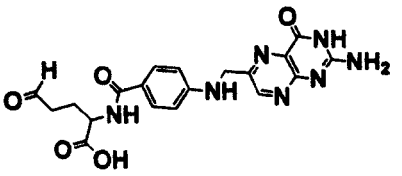
Poly(ethylene glycol) methyl ether methacrylate



Glycolide



Propargyl amine



Folic acid

4.2. Materials and Methods

4.2.1. Instrumentation

Number average molecular weight (*Mn*), weight average molecular weight (*Mw*) and polydispersity Index (PDI= *Mw*/*Mn*) of the prepared copolymers were obtained by Gel Permeation Chromatography (GPC) (PL-120 and PL-50 Polymer Labs) with a refractive index detector. The columns (7.5 x 300 mm Resipore Mixed-D, 2 in series) were eluted by chloroform and calibrated with polystyrene standards. All calibration and analysis were performed at 40 °C and a flow rate of 1 mL min-1. All of the products were dissolved in chloroform and passed through 0.2 μm filter prior to column chromatography.

^{1}H NMR spectra were recorded at 20 °C on a Bruker-DRX instrument operating at 400 MHz. Chemical shifts (δ) are reported in parts per million (ppm), referenced to $CDCl_3$ (δ ppm = 7.26). The data was processed using the TOPSPIN 2 software.

Infrared spectra were measured on an Avatar 360 Nicolet FT-IR spectrophotometer in the range of 4000-500 cm^{-1} by preparation of KBr discs. Sample analyses were carried out by the E2 OMNIC software for analysis. Mass spectra (TOF-ES) were recorded on a Waters 2795 separation module/micromass LCT platform.

Values of hydrodynamic radius of the nanoparticles were determined via scattered light recorded at 90° angle to incident radiation in Dynamic Light Scattering (DLS) using a Viscotec Model 802 instrument equipped with an internal laser (825-832 nm) with a maximum radiation power of 60 mW. The samples were diluted with filtered, deionised water and at least five measurements of each sample were taken. The mean and standard deviation were calculated. Data processing was performed with the software program OmniSize2.

Zeta potential (ξ) measurements of the nanoparticles were performed by laser Doppler anemometry using a Malvern Zetasizer 2000 equipped with a 10 mW He-Ne laser operating at a wavelength of 633 nm. Measurements were performed in low ionic strength buffer, 1.0 mM Phosphate buffer adjusted to pH 7.4. The mean value and standard deviation for each sample was calculated from at least five measurements.

Morphology of the particles was examined using Transmission Electron Microscope (TEM) (Jeol Jem 1010 electron microscope, Japan)c. A sample of particle suspension was diluted with a solution of phosphotungstic acid (3% w/v, pH 7.4) and observed under TEM. One drop of sample was placed for 1 minute on a copper grid coated with a formvar carbon film. The excess of sample was wicked away with the aid of filter paper prior to imaging by TEM.

All solvents and reagents were of analytical or HPLC grade and purchased from Sigma or Fisher Scientific unless otherwise stated. Deuterated solvents were from Sigma. The two monomers DL-lactide and glycolide were obtained from Purac biochem. The monomers were recrystallized from ethyl acetate prior to use. Polyethylene glycol methyl ether (PEGMA-ME 475, *Mn* = 475) were purchased from Sigma Aldrich and purified before use by passing through a column filled with neutral alumina. Copper (II) bromide (CuBr2, 99%), 2-bromo-2-methylpropionyl bromide, L-ascorbic acid (99%), N, N, N', N'', N'''-Pentamethyldiethylenetriamine (PMDTA, 98%) and tin (II)- 2-ethylhexanoate were used as received from Sigma Aldrich. N, N-azobis (isobutyronitrile) (AIBN, 98%, Aldrich) was recrystallized from ethanol. Plasmid DNA gWIZ-luc, containing the firefly luciferase gene was supplied as 5mg/ml solution by Aldevron (South Fargo,USA) and used without further treatment.

The luciferase detection kit was acquired from Promega (Southampton, UK) and contained cell culture lysis reagent (5 x solutions), Luciferin reagent and Luciferase assay buffer. Phosphate Buffer Saline (PBS) was used as received from Fisher Scientific. Dialysis membrane (MWCO 3500, regenerated cellulose) was used as received from Spectrapor.

4.3. Polymer syntheses

4.3.1. Synthesis of 2-bromo-2-methyl-propionic acid 2-(2-hydroxy-ethyldisulfanyl)-ethyl ester (2)

The heterobifunctional ATRP/ROP initiator was prepared as follows. Dithiodiethanol (12 g, 0.077 moles) was added to a 100ml round bottom flask equipped with three-way stopcocks connected to either an argon line or a vacuum pump, and dried via azeotropic distillation with anhydrous toluene (3X 20ml) under reduced pressure. After complete evaporation of toluene, anhydrous THF (20ml) was added via a syringe under an argon atmosphere. Triethylamine (2.3 g, 0.023 moles) was added to the solution which was maintained at 4 °C during slow addition of α-bromoisobutyryl bromide (2.6 g, 0.011 M). The solution was allowed to warm to room temperature and left overnight. The solution was then filtered to remove the salt and the crude product was recovered by evaporation of THF. The product was purified by passing through silica column chromatography using ether as eluent (yield 90%)

^{1}H NMR ($CDCl_3$, 400 MHz)): 1H NMR (CDCl3, 400 MHz): δ = 4.48-4.44 (t, j= 6.68 Hz, 2H, CH2-OH)., 3.92-3.8 ($q_{apparent}$, j_{app} = 5.94 Hz, 2H, CH2-O-C=O), 3-2.97 (t, J = 6.62 Hz, 2H, CH2-S), , 2.91-2.89(t, J= 5.835, 2H, CH2-S), 2.1(t, J = 6.185,H). 1.95 (s, 6H, CH3).

^{13}C NMR ($CDCl_3$): δ = 171.9, 63.7, 60.4, 55.6, 41.5, 36.6, 30.8. HR-MS (ES+) calcd for C8H15BrO3S2 (MH+) 301.965 and 303.237, found 301.8102 and 303.8056. FTIR (cm^{-1}) 3410, (-OH), 2927, (C-H), 1734, (C=O).

4.3.2. Synthesis of PLGA polymer using 2-bromo-2-methyl-propionic acid 2-(2-hydroxy-ethyldisulfanyl)-ethyl ester (P 9)

PLGA, Poly (DL-lactide-co-glycolide) was synthesized by ROP (ring opening polymerization) using 2-((2-hydroxyethyl) disulfanyl) ethyl 2-bromopropanoate as initiator. First, DL-lactide (1.82 g, 0.012 moles), glycolide (1.47 g, 0.012 moles), and 2-((2-hydroxyethyl)disulfanyl)ethyl 2-bromopropanoate (0.4g, 0.0014 moles) were added to a polymerization tube and purged with argon several times before being heated in an oil bath to 80 °C and purged with argon for a further 2 hours. The reaction mixture was then heated to 140 °C in order to conduct the polymerisation in the melt phase. At this point, tin (II) 2-ethylhexanoate (0.106 g, 0.00027 moles) was added, and the mixture was stirred for 24 hours. The polymerization tube was then removed from the oil bath and cooled down to room temperature. The polymer was recovered by dissolution in THF and precipitated in methanol three times to remove unreacted monomer. The precipitate was filtered and dried under reduced pressure to give a white product (yield 95%). The molecular weight of the polymer and poly dispersity index was determined by gel permeation chromatography using chloroform as eluent.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H, CH2-C=O), 4.2-4.3(m, 4H, CH2-O- C=O), 2.8-2.9 (m, 4H, CH2-S), 1.8-1.9 (s, 6H, CH3), 1.58 (d, 3H, CH3-CH). ^{13}C NMR ($CDCl_3$): δ = 169.5, 166.3, 69.1, .60.8, 53.5, 16.9. FTIR (cm^{-1}) 2860–2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

4.3.3. Synthesis of PLGA-S-S-PEGMA$_{475}$ block copolymer using (P 10)

The PLGA-S-S-PEGMA$_{475}$ block copolymers were prepared by Atom Transfer Radical Polymerization (ATRP) using the PLGA-S-S-Br block as macro-initiator For PLGA-S-S-PEGMA$_{475}$ block copolymers, PLGA-S-S-Br (1.5 g, 0.0003 moles, Poly(ethylene glycol)methyl ether methacrylate 475 (2.8 g, 0.0063 moles), CuBr2 (0.067 g, 0.0003 moles) and PMDTA (63 μL, 0.0003 moles) were added into a round bottom flask, the flask purged with argon several times, followed by addition of butanone (10ml) and degassed for 20min under an argon atmosphere. L-ascorbic acid (0.022 g, 0.00012 moles) was added to the polymerisation solution under argon condition. The mixture was then heated in oil bath to 50 °C and stirred for 3.5 hours. The polymerization product was precipitated in a mixture of hexane/ether (50:50), and then the polymer was passed through a short alumina column to remove copper salts. Finally, the solvent was evaporated and the product dried under reduced pressure (yield 85%). The product was characterized by NMR and GPC using chloroform as eluent.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H,CH2- C=O), 3.94-4.14 (m, CH2, CH2-O- C=O), 3.8-3.4 (m, CH2CH2O), 3.2-3.38 (S,-CH3), 1.58 (s, 3H, CH3), 1.6-1.9(m,CH2), 0.7-1.3 (s,CH3). ^{13}C NMR ($CDCl_3$): δ = 169.5, 166.3, 71.9, .70.5, 59.2, 16.9 FTIR (cm^{-1}) 2860-2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

4.3.4. Synthesis of Azide-Terminated PLGA-S-S-PEGMA$_{475}$ block copolymer (P 11)

Sodium azide (0.04 g, 0.00062 moles) was added to a round-bottom flask containing PLGA-S-S-PEGMA$_{475}$ block copolymer (4 g, 0.0003 moles) dissolved in DMF (12 mL). The reaction mixture was stirred at

50 °C for 24 hours after which time it was allowed to cool down to room temperature, After elimination of DMF under reduced pressure, toluene (15 mL) was added, and the insoluble salt was removed by centrifugation (10000 rpm at 25 °C for 25 min). Finally, the toluene was evaporated under reduced pressure (yield 95%). The product was characterized by FTIR, 1H-NMR and GPC.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H,CH2- C=O), 3.94-4.14 (m, CH2, CH2-O- C=O), 3.8-3.4 (m, CH2CH2O), 3.2-3.38 (s,CH3), 1.58 (s, 3H, CH3), 1.6-1.7(m, CH2-N3), 0.7-1.3 (s,CH3). FTIR (cm^{-1}) 2860-2940 (C-H), 2106 (N3), 1760 (C=O).

4.3.5. Synthesis of Propargyl Folate (3)

This was accomplished by a method modified from the literature[21]. Folic acid (1.0g, 0.0022 moles) was dissolved in DMF (10 mL) and cooled in a water/ice bath. N-Hydroxysuccinimide (0.26 g, 0.0025 moles) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, 0.44 g, 0.0025 moles) were added, and the resulting mixture was stirred in an ice bath for 30 min to give a white precipitate. A solution of propargylamine (0.124 g, 2.25 moles) in DMF (5.0 mL) was added, and the resulting mixture was allowed to warm to room temperature and stirred for 24 h. The reaction mixture was poured into water (100 mL) and stirred for 30 min to form a precipitate. The orange-yellow precipitate was filtered, washed with acetone, and dried under vacuum to give a product in (yield 90%). Analytical data corresponded to those of the literature reported product.

^{1}H NMR (DMSO-d6, 400 MHz): 8.64 (s,1H, PtC7 H), 8.29-8.24 (d,1H, PtC6-CH2NH-Ph), 8.04-8.02 (d,1H, -CONHCHCO2H), 7.67-7.65 (d, 2H ,Ph-C2H and Ph-C6H) 6.93 (br s, 2H, NH2, 2H), 6.65-6.63 (d,2H, Ph-C3H and Ph-C5H), 4.49-4.48 (d,2H, PtC6-CH2NH-Ph), 4.32-4.30 (m,1H,-CONHCHCO2H), 3.84-3.81 (m,2H, -CONH-CH2C≡CH), 3.07-3.05 (t, 1H, -CONHCH2C≡CH), 2.88 (s, 1H, -CONH-CH2C≡CH,), 2.72 (br s,1H, OH,), 2.31-2.20 (m, , 2H, -CH2CO2H), 1.98-1.96 (m,1H, -CHCH2CH2), 1.87-1.85 (m,1H, -CHCH2CH2). Note: Pt = pteridine.

4.3.6. 'Click' reaction of azide terminal of PLGA-S-S-PEGMA$_{475}$ blocks copolymer with propargyl folate (P 12) and propargyl alcohol (P 13)

In a 25ml round bottom flask, PLGA-S-S-PEGMA$_{475}$-N$_3$ (2 g, 0.0002 moles, Mn = 9000 g/mol), PMDTA (0.034 g, 0.00002 moles) and propargyl folate (0.014 g, 0.00024 moles) were dissolved in DMF (10ml) and purged with argon for 30 minutes. The solution was then transferred via a degassed canulla needle to another tube equipped with a magnetic stir bar containing CuBr (0.028 g, 0.0002 moles) under argon atmosphere. The mixture was stirred at 26 °C for 24 h in the absence of oxygen. After the reaction, the mixture was exposed to air, and the solution was passed through a column of neutral alumina. DMF was removed under vacuum. The resulting folate-terminated PLGA-S-S-PEGMA$_{475}$ was dissolved in THF and filtered to remove the excess of propargyl folate. The product was further purified by dialysis in acetone using regenerated cellulose membrane molecular cut off of 3500 Da. Finally, the polymer was dried under vacuum for 6 hours and the product was characterized by FTIR, ^{1}H-NMR and GPC (yield 80%). The synthesis of (P 13) was exactly analogous except propargyl alcohol was used in place of propargyl folate (yield 82%).

P 12: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.5, 8.2, 7.5, 6.5 (weak multiplets, folate terminus), 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H,CH2- C=O), 3.94-4.14 (m, CH2, CH2-O- C=O), 3.8-3.4 (m, CH2CH2O), 3.2-3.38 (S,-CH3), 1.58 (s, 3H, CH3), 1.6-1.9(m,CH2), 0.7-1.3 (s,CH3). ^{13}C NMR ($CDCl_3$): δ = 169.5, 166.3, 71.9, .70.5, 59.2, 16.9. FTIR (cm^{-1}) 2860-2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

P 13: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H,CH2- C=O), 3.94-4.14 (m, CH2, CH2-O- C=O), 3.8-3.4 (m, CH2CH2O), 3.2-3.38 (S,-CH3), 1.58 (s, 3H, CH3), 1.6-1.9(m,CH2), 0.7-1.3 (s,CH3).). ^{13}C NMR (CDCl3): δ = 169.5, 166.3, 71.9, .70.5, 59.2, 16.9. FTIR (cm^{-1}) 2860-2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

4.3.7. Synthesis of PLGA-S-S-PLGA homo-polymer, using 2, 2-Dithiodiethanol as bifunctional initiator (P 14)

PLGA-S-S-PLGA homopolymer, Poly (DL-lactide-co-glycolide-S-S-DL-lactide-co-glycolide) was synthesized by ROP (ring opening polymerization) using dithiodiethanol (2) as bifunctional initiator. First, DL-lactide (2.7g, 0.018 moles), glycolide (2.1 g, 0.018 moles), and dithiodiethanol (0.3g, 0.0019 moles) were added to a polymerization tube and purged with argon several times before being heated in an oil bath to 80 °C and purged with argon for a further 2 hours. The reaction mixture was then heated to 140 °C in order to conduct the polymerisation in the melt phase. At this point, tin (II) hexanoate (0.078g, 0.0002 moles) was added, and the mixture was stirred for 24

hours. The polymerization tube was then removed from the oil bath and cooled down to room temperature.

The polymer was recovered by dissolution in THF and precipitated in methanol (this was repeated three times to remove unreacted monomer). The precipitate was filtered and dried under reduced pressure to leave a white solid product (yield 94%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.18 (q, 1H, CH- C=O), 4.82 (q, 2H, CH2- C=O), 4.36-4.4 (m, 4H, CH2-O- C=O), 2.87-2.96 (m, 4H, CH2-S), 1.58 (d, 3H, CH3-CH). ^{13}C NMR ($CDCl_3$): δ = 169.5, 166.3, 69.1, .60.8, 53.5, 16.9. FTIR (cm^{-1}) 2860-2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

4.3.8. Synthesis of PLGA-TEG-PLGA homo-polymer, using tetraethylene glycol (TEG) as bifunctional initiator (P 15)

Poly (DL-lactide-co-glycolide-TEG-DL-lactide-co-glycolide) was synthesized by ROP (ring opening polymerization) using tetraethylene glycol initiator (3). First, DL-lactide (2.1g, 0.014 moles), glycolide (1.7 g, 0.014 moles), and tetraethylene glycol (0.3g, 0.0015 moles) were added to a polymerization tube and purged with argon several times before being heated in an oil bath to 80 °C and purged with argon for a further 2 hours. The reaction mixture was then heated to 140 °C in order to conduct the polymerisation in the melt phase. At this point, tin (II) 2-ethylhexanoate (0.062g, 0.00015 moles) was added, and the mixture was stirred for 24 hours. The polymerization tube was then removed from the oil bath and cooled down to room temperature. The polymer was recovered by dissolution in THF and precipitated in methanol (this was repeated three times to remove unreacted monomer). The precipitate was filtered and dried under reduced pressure to yield a white solid product (yield 93%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.18 (m, 1H, CH- C=O), 4.82 (m, 2H,CH2- C=O), 4.27-4.34 (m, 2H, CH2-O- C=O), 3.6-3.65 (s, 8H, CH2CH2-O), 3,67-3.72 (m, 4H, CH2CH2-O- C=O), 1.58 (d, 3H, CH3-CH). ^{13}C NMR ($CDCl_3$): δ = 169.5, 166.3, 69.1, .60.8, 53.5, 16.9. FTIR (cm^{-1}) 2860–2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

4.4. Fabrication of non-loaded nanoparticles

Nanoparticles with PLGA-S-S-PLGA (P14): PLGA-S-S-$PEGMA_{475}$ (P12) ratios of 50:50 were prepared using a modified solvent diffusion technique. P12 and P14 50mg of each were dissolved in of dichloromethane (2 ml). The organic solution was then mixed by vortex agitation for 30 seconds with an aqueous phase (200 μL). The obtained emulsion was then poured into a polar phase (25 ml of ethanol) under moderate magnetic stirring, resulting in an immediate precipitation of polymer nanoparticles. The suspension was diluted with ultra-pure water (25 ml) and stirred for 1 hour. The organic solvent was evaporated under reduced pressure at 300C. The final particles solution was passed through a Sephadex column (PD 10) and then characterised by dynamic light scattering (DLS), Zeta Potential measurements and transmission electron microscopy (TEM).

4.4.1. Characterisation of nanoparticles

4.4.1.1. Dynamic light scattering

The size and polydispersity of nanoparticle suspensions were determined by dynamic light scattering. An aliquot was added in a quartz cuvette and the sample was examined on a Viscotek 802 dynamic light scattering instrument.

Scattered light from a 50 mW laser source (830 nm) was recorded from an internal light detector aligned at 90o from source. From standard auto correlation functions, measured correlation coefficients were related to hydrodynamic radii at varying temperatures via the Stokes-Einstein equation, $RH = KT/ 6\pi\eta D$, where RH is the hydrodynamic radius, k is the Boltzmann constant, T is the temperature and η is the viscosity of the solvent. The size distribution of the scattering particles was determined by the average mass number of the particles.

4.4.1.2. Surface charge measurement (Zeta Potential)

The surface charge of the particle was measured on a Malvern Instruments Zetasizer 2000. To determine the zeta potential, the measurements were performed in 1mM phosphate buffer solution at pH 7.4. Mean values were obtained from three different batches, each measured three times.

4.4.1.3. Microscopy

The morphology of the nanoparticles was examined by Transmission Electron Microscopy (TEM). Samples were imaged using a JEOL (JEM-1010) electron microscope. A few drops were added onto the copper grid and allowed to dry in air. The particles were negatively stained with 3% phosphotungstic acid solution.

4.4.2. Determination of plasmid DNA encapsulation efficiency

The theoretical loading of plasmid DNA was 0.4% (w/w) in regards to the total amount of P14 in the nanoparticles. For the determination of the actual amount of encapsulated DNA, an extraction method was used. Briefly, nanoparticles (10mg), loaded with plasmid DNA were re-suspended in 0.5 N NaOH (1.5 ml) under horizontal shaking at 37 °C until a clear solution was obtained. The DNA content in the resulting solution was quantified by UV spectrophotometry (Shimadzu 1204, Tokyo, Japan) at 260nm by means of a standard absorption curve derived from DNA in water. The encapsulation efficiency was calculated as the ratio between the actual and theoretical DNA loading percentages. Each experiment was performed in triplicate.

4.4.3. Release profile of plasmid DNA from the nanoparticles

The in vitro release of DNA in presence/absence of glutathione (as a representative intracellular reducing agent) was investigated as follows. Nanoparticles (10 mg) were suspended in glutathione solution (1.5 ml, 5mM) at 37 °C under continuous agitation. At various time intervals, the supernatant was withdrawn and fresh buffer was replenished. The amount of plasmid DNA in the supernatant was determined by UV spectrophotometry at 260 nm. For control experiments, phosphate-buffered saline (PBS) pH 7.4 was used instead of glutathione solution and non-reducible PLGA nanoparticles were also studied in the presence of GSH and PBS. The experiments were performed in triplicate.

4.4.4. Cell toxicity assay

MTS assay was performed to evaluate the effect of nanoparticles on cell metabolic activity. Calu-3 cells were seeded on 96-well plates at a density of 10,000 cells per well and cultured in the EMEM medium. Prior to the test, cell medium was removed and replaced by sample solution comprising of nanoparticles in HBSS. The samples contained (4mg.mL^{-1}) concentration of nanoparticles. 0.1% Triton-X 100 and HBSS were used as a positive and negative control, respectively.
Cells were incubated under standard conditions with the samples or the controls for a period of two hours, after which they were removed. Cells were then washed with PBS following which the MTS assay was performed according to the manufacturer's instructions.

4.4.5. Cellular uptake experiments

Prior to the experiment, the culture medium was removed, the cell monolayers rinsed with phosphate buffer saline (PBS) and then allowed to equilibrate for 1h at 37 °C in Hanks Balanced Salt Solution (HBSS) with 15mM 4(2hydroxyethyl)-1piperazine-ethane sulfonic acid (Sigma). A series of batches of nanoparticles were prepared, varying in the mass ratio of P12: P14 from (5% to 75%) and a batch of P13: P14 of 75% mass ratio (used as control). All the batches were labeled with Coumarin 6 as a label to assess the cellular uptake. Nanoparticles samples composed of (2mg.mL^{-1}) concentrations of nanoparticles suspended in HBSS buffer were applied and the uptake study performed for a period of four hours. The samples were then removed and the cell monolayers were washed with buffer. In the next stage, the cells were digested with 0.2 N NaOH in 0.5% Triton X-100 and centrifuged at a speed of 13000 rpm for 30 sec (Eppendorf 5417 R centrifuge-AG 22331 Hamburg).

The fluorescence intensity of the supernatant was measured by Fluorescence Spectrophotometer (MFX Microtiter Plate Fluorometer, The Microtiter Company, and UK).

For inhibition experiments, 1mmol/L free folate was added prior to application of nanoparticles.

4.4.6. Transfection study

Twelve well plates were seeded with 1 x 10^5 cells per well and the cells were incubated at 37 °C and under atmosphere of 5% CO_2 for 24 h. After 24 h, the medium was aspirated off from the wells and gently replaced with 1ml DNA-loaded nanoparticles. A volume of nanoparticles containing 4µg of DNA were prepared as described above. The cells were incubated for 4 h at 37 °C and 5% CO2. Following this incubation, the nanoparticles were removed and replaced with RMPI-1640 medium and the cells were incubated for a further 24 h before analysis.

4.4.6.1. Detection of luciferase

The medium was removed from the cells, which were then washed with PBS twice. Luciferase detection was performed with a luciferase detection kit. Cell lysis buffer (300 µL) was added to each well. A pipette tip was used to scrape all cells from the well substrate, after which the lysate was centrifuged at 13,500 rpm for 5 minutes and the supernatants were collected. The luciferase activity of each sample was measured by gently mixing reconstituted luciferin reagent (100 µL) with cell lysate (20 µL) in a scintillation vial insert, and inserting into a luminometer (TD 20/20, Progema).

4.4.6.2. Determination of cellular protein

Cellular protein contents were assayed by the Bradford method. Bovine serum albumin (BSA) standards were prepared from a 1 $mg.mL^{-1}$ stock solution by diluting with one-10th strength lysis buffer to 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 $mg.mL^{-1}$. Aliquots of each standard (10 µl) were transferred into a 96-assay plate, then 200 µl Bradford assay reagent was added and the A_{595} was measured after 15 minutes. From samples prepared in the luciferase detection protocol, 20 µl of each sample was placed in an eppendorf and diluted with 180 µl of water (interference from lysis buffer, need to dilute 10x). Again aliquots of 10 µl were transferred to the wells of a 96-well assay plate and 200 µl Bradford reagent was added. The mixtures were allowed to stand for the same time as the standards and then the A_{595} measured. A standard curve was prepared and a linear curve fit used to give the equation relating A_{595} to protein concentration, which was employed to calculate the protein concentration in the samples. Results in light units/ml obtained from previous sections were divided by mg protein/ml from this assay to give a final value of light units/mg cellular protein.

4.5. Results and Discussion

The concept underlying the work was the generation of surface functionalized multi-component core-shell drug delivery systems, containing chemistries to enable cell surface receptor targeting, prevention of extracellular protein adsorption and opsonisation, intracellularly triggered loss of the polymer shell and ultimately, biodegradation. In addition, the plan was to combine block components such that the co-polymers could assemble into well-defined nanoparticles in water/oil/water (w/o/w) emulsions, in order to encapsulate and protect sensitive biopolymers such as DNA. Accordingly, block copolymers were chosen of poly (DL-lactic-co-glycolic acid) (PLGA – the hydrophobic core block) and (polyethylene glycol methyl ether) ($PEGMA_{475}$ - the hydrophilic outer block), connected by a bioreducible disulfide bridge. In addition, facile derivatisation of the hydrophilic block terminus was required to install a ligand for cell receptor targeting. The synthetic strategy therefore combined two different polymerisation techniques, ring opening polymerisation (ROP) and Atom Transfer Radical Polymerisation (ATRP), using an initial heterobifunctional initiator 2-bromo-2-methyl-propionic acid 2-(2-hydroxy-ethyldisulfanyl)-ethyl ester (1), followed by well-established 'click' chemistries as shown in Figure 4-2.

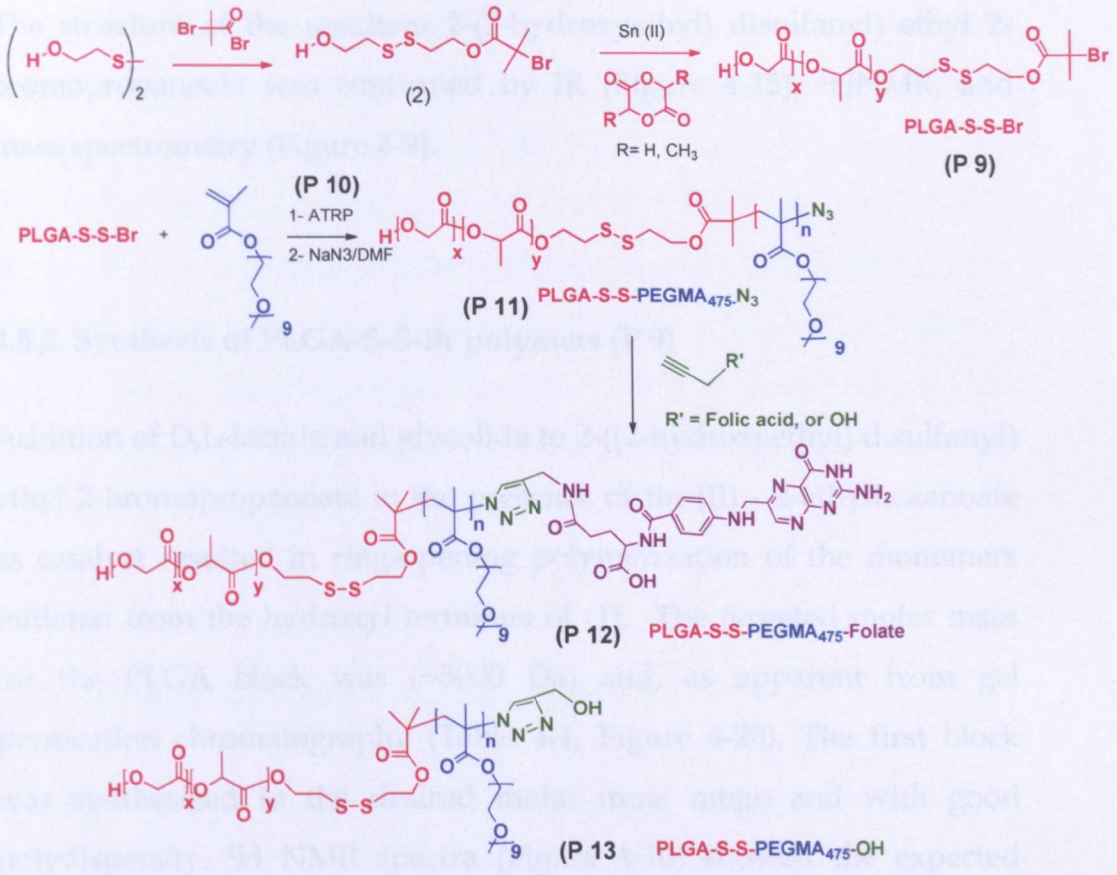


Figure 4-2. Synthetic strategy for end-functional block co-polymers.

4.5.1. Synthesis of (2-bromo-2-methyl-propionic acid 2-(2-hydroxy-ethyldisulfanyl)-ethyl ester) (2)

A heterobifunctional unit was required to initiate the two polymerization route i.e. ROP for the synthesis of the core PLGA block and ATRP for the synthesis of the polyPEGMA$_{475}$ exterior block. The link between the two blocks needed to be cleavable, for which a disulfide bridge was chosen as reduction of disulfides by intracellular glutathione (GSH) has been previously described. Compound (2) was accordingly obtained by esterification of dithiodiethanol with 2-bromo-2-methylpropionyl bromide.

The structure of the resultant 2-(2-hydroxyethyl) disulfanyl) ethyl 2-bromopropanoate was confirmed by IR (Figure 4-15), ^{1}HNMR, and mass spectrometry (Figure 4-9).

4.5.2. Synthesis of PLGA-S-S-Br polymers (P 9)

Addition of D,L-lactide and glycolide to 2-((2-hydroxyethyl) disulfanyl) ethyl 2-bromopropanoate in the presence of tin (II) - 2-ethylhexanoate as catalyst resulted in ring-opening polymerization of the monomers initiated from the hydroxyl terminus of (1). The targeted molar mass for the PLGA block was (~5000 Da) and, as apparent from gel permeation chromatography (Table 4-1, Figure 4-20). The first block was synthesized in the desired molar mass range and with good polydispersity. ^{1}H NMR spectra (Figure 4-10) showed the expected resonances of the polymer backbone at 5.1 ppm and 1.57 ppm (methine and methyl protons of poly (lactic acid), and at 4.8 ppm (methylene group of poly (glycolic acid).

4.5.3. Synthesis of PLGA-S-S-PEGMA$_{475}$ block copolymer (P 10)

Atom Transfer Radical Polymerisation (ATRP) of PEGMA$_{475}$ from the 2-bromo-2-methyl-propanoate-tipped PLGA was carried out using the AGET ATRP method[36, 37]. The polymerization was stopped at low conversion to control polydispersity and ensure a high degree of bromine end-functionality for subsequent derivatisation. The polymerisation of the second block was confirmed by GPC (Figure 4-20) which showed a decrease in retention time of the PLGA-S-S-PEGMA$_{475}$ in comparison to PLGA-S-S-Br.

The GPC traces were unimodal, indicating successful synthesis of the second block from the first block via the ATRP process. The molar mass distribution of the PLGA-S-S-PEGMA$_{475}$ block copolymer was essentially the same as that of the precursor PLGA block which per se demonstrated controlled polymerisation for the second component. The structure of the diblock copolymer was confirmed by ^{1}H NMR spectra. (Figure 4-11) and the composition and ratios of each block in the copolymer were determined by relative peak integrals of PLGA and PEGMA proton resonances.

4.5.4. Synthesis of PLGA-S-S-PLGA (P 14)

A reducible 'homo-diblock' PLGA polymer, designed to act as a bioresponsive core of the particles, was synthesized from dithiodiethanol and DL-lactide and glycolide monomers via tin (II)-2-ethylhexanoate catalysed ROP as before. The targeted molar mass weight was again ~5.000 Da, but in this case the copolymer grew from both ends of the dithiodiethanol initiator. GPC traces (Figure 4-20) showed relatively good control over polydispersity of the polymer, with the unimodal feature of the GPC traces suggesting successful progressive chain extension on either side of the initiator the structure of the diblock was also confirmed by spectroscopy.

4.5.5. Synthesis of Azido-and Folate Terminated PLGA-S-S-PEGMA$_{475}$ diblock copolymers (P 11 and P12)

The bromine-terminated PLGA-S-S-PEGMA$_{475}$ diblock copolymer was transformed into an azide-terminated diblock copolymer via nucleophilic substitution with sodium azide[38, 39]. The molar mass of the azide-terminated PLGA-S-S-PEGMA475 was unchanged compared to the bromo-precursor as indicated by GPC (Figure 4-20).

The ^{1}H NMR spectrum (Figure 4-12) was similar to that of the bromide terminated block copolymer with the exception of the methylene residue at the terminus: this resonance shifted from 1.9 ppm in the Br-terminated polymer to 1.7 ppm in the azide-derivative suggesting a complete substitution of the terminal bromine by azide. FT-IR spectra confirmed the presence of azide functionality through a strong absorption band at ~2110 cm-1. (Figure 4-18) The final step in the synthetic procedure involved modification of the terminal azide via copper-mediated 'click' coupling with an acetylene-tipped folic acid derivative, propargyl folate in DMF at room temperature. N, N', N'', N''', N''''-Pentamethyldiethylenetriamine (PMDTA) was employed to enhance reaction rate and increase end-terminal modification. GPC again confirmed no change in molar mass occurred during the "click" reaction (Figure 4-20). FT-IR demonstrated the loss of azide group absorption at 2110 cm-1 (Figure 4-19) and 1H NMR showed the presence of folate aromatic protons at δ =6-8ppm (Figure 4-14). UV absorptions at 363 nm, representative of folate provided further support for the presence of azide functionality (Figure 4-23).

The number of folate residues per diblock copolymer of PLGA-S-S-PEGMA475 was determined to be ~ 1 as expected. The properties of the co-polymers are summarized in Table 4-I.

Table 4-I. Properties of polymers and diblock copolymers using ROP and ATRP methods

Polymer	Mn_{theo} x10^{3a}	Mn x10^{3b}	Mw x10^3	Mw/Mn
PLGA-S-S-Br (P 9)	4.9	4.9	6.5	1.3
PLGA-S-S-PEGMA475.Br (P 10)	9	8.4	10.2	1.2
PLGA-S-S-PEGMA475.N3 (P 11)	9	8.1	9.7	1.2
PLGA-S-S-PEGMA475.Folate(P12)	9	9.4	12	1.3
PLGA-S-S-PEGMA475-OH (P 13)	9	9.4	12	1.3
PLGA-S-S-PLGA (P 14)	4.9	4.2	5	1.2
PLGA-TEG-PLGA (P 15)	4.9	4	4.7	1.2

[a] Theoretical, from monomer/initiator ratio. [b] From GPC (CHCl3) ,30 °C, poly(styrene) standards).

4.6. Fabrication of nanoparticles

The PLGA-S-S-PEGMA$_{475}$ and PLGA-S-S-PLGA block copolymers were then used to prepare blend matrix nanostructures as shown in Figure 4-3.

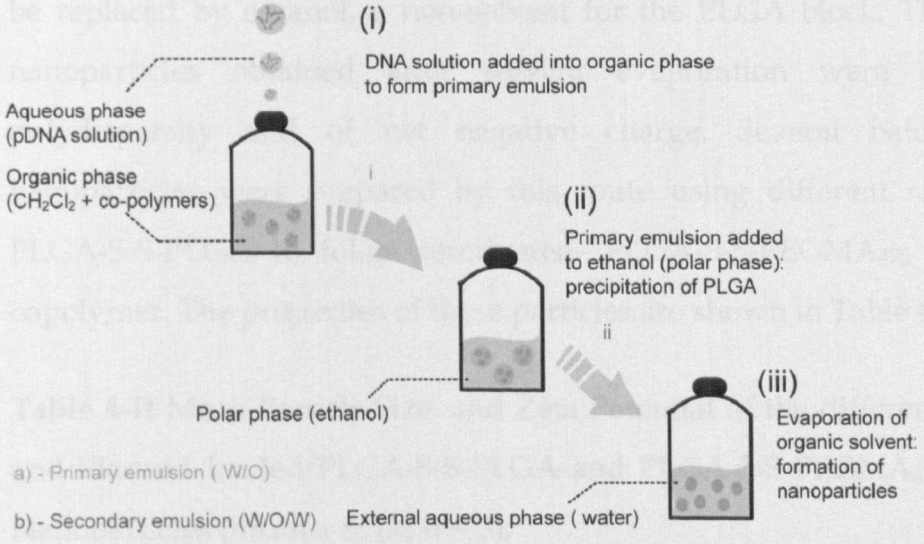


Figure 4-3. Preparation of core-shell nanoparticles using a w/o/w emulsion technique. In (A) DNA in water is added into dichloromethane (DCM) containing PLGA-S-S-PEGMA$_{475}$ polymers. Addition of this primary emulsion to ethanol (B) results in collapse of the hydrophobic core as the DCM partitions into the bulk phase. Solvent evaporation (C) yields the final nanoparticles.

The double emulsion technique was selected to encapsulate DNA as the formation of nanoparticles by this route occurs via solvent diffusion, under conditions that are mild and which avoid the shear-induced degradation of DNA prevalent during conventional nanoparticle formation. To accomplish this, an aqueous solution of plasmid DNA was added into dichloromethane (organic phase) containing PLGA-S-S-PLGA and folate terminated PLGA-S-S-PEGMA$_{475}$.

The primary emulsion needed only a mild vortex to form, which was most likely to have been a result of the amphiphilic nature of the folate terminated PLGA-S-S-PEGMA$_{475}$. Addition of this emulsion to a more polar phase (ethanol) resulted in the immediate formation of nanoparticles as dichloromethane diffused from the inner oil phase to be replaced by ethanol, a non-solvent for the PLGA block. The final nanoparticles obtained after solvent evaporation were of low polydispersity and of net negative charge. Several batches of nanoparticles were prepared by this route using different ratios of PLGA-S-S-PLGA to folate terminated- PLGA-S-S-PEGMA$_{475}$ diblock copolymer. The properties of these particles are shown in Table 4-II.

Table 4-II Mean Particle Size, and Zeta Potential of the different Blank and Plasmid loaded PLGA-S-S-PLGA and PLGA-S-S-PEGMA$_{475}$-folate Nanoparticles (mean ± S. D., n = 3).

Batch	Ratio [a]	Blank Mean size(nm) [b]	Plasmid-loaded Mean size(nm)	Blank-Zeta Potential (mV)	Plasmid loaded Zeta Potential (mV)
NPS1	95:5 [c]	148±1	.....	-15.63±0.25	.....
NPS2	90:10 [c]	145±1.6	.....	-14.13±0.05	.....
NPS3	75:25 [c]	128±3.6	.....	-6.93±0.37	.....
NPS4	50:50 [c]	108±4	96.2±1	-7.2±0.17	-9.9
NPS5	25:75 [c]	95±3.1	.....	-4.5±0.40	.....
NPS6	50:50 [d]	110±4.7	.....	-13.3±0.26	.....
NPS7	25:75 [d]	139±2.7	.....	-15.10±0.17	.....
NPS8	50:50 [c1]	111±1.4	98±2.7	-8.5	-9.5
NPS9	50:50 [d1]	107.6±1	102.1±1	-13.1	-13.6

[a] The (wt/wt %) ratio of PLGA-S-S-PLGA :PLGA-S-S-PEGMA475-folate, [b] mean diameter size based on relative mass distribution. [c]; - derived from P3a (folate-terminated), [d] - derived from P3b (hydroxyl-

terminated). [c1,d1] both are derived same as [c, d] but in these two formulation P5 (PLGA-TEG-PLGA) was used instead of P4

As apparent from Table 4-II, increasing the $PEGMA_{475}$-folate: PLGA-S-S-PLGA ratio (NPS1-5) decreased both particle size and zeta potential. This was due to the greater interfacial stabilization during primary emulsion formation as the $PEGMA_{475}$ content was increased, and the shielding of PLGA-carboxyl groups as the $PEGMA_{475}$-folate 'corona' was enlarged. Particle sizes were similar for NPS4 and NPS6, which contained PLGA-S-S-$PEGMA_{475}$-folate: PLGA-S-S-PLGA (50:50 mole %), and PLGA-S-S-$PEGMA_{475}$-OH: PLGA-S-S-PLGA (50:50 mole %), respectively, at ~ 110 nm, when DNA was not encapsulated. Similarly, NPS8 and NPS 9, which were of the same molar ratio of PLGA-S-S-$PEGMA_{475}$-folate or PLGA-S-S-$PEGMA_{475}$-OH but with a non-reducible PLGA-TEG-PLGA in place of PLGA-S-S-PLGA, were formed as ~110nm particles in the absence of DNA. However, for NPS4, 8 and 9, the encapsulation of DNA decreased both particle size and zeta potentials. These differences in size and charge of the particles containing DNA indicate that the nucleic acids may also have played a role in stabilizing the initial o/w interface in the primary emulsion.

Microscopy of nanoparticles (3% phosphotungstic acid negative staining) showed consistent spherical shapes across the compositional ranges, Figure 4-4. Particle sizes determined from TEM micrographs were slightly less than the hydrodynamic diameters recorded by dynamic light scattering (DLS), most likely due to air-drying of the nanoparticles on the copper grid leading to collapse of hydrated ($PEGMA_{475}$) segments on the outer shells. The apparent particle size range by TEM was higher than observed in DLS, which may reflect the relative insensitivity of light scattering techniques in detecting smaller compared to larger particles.

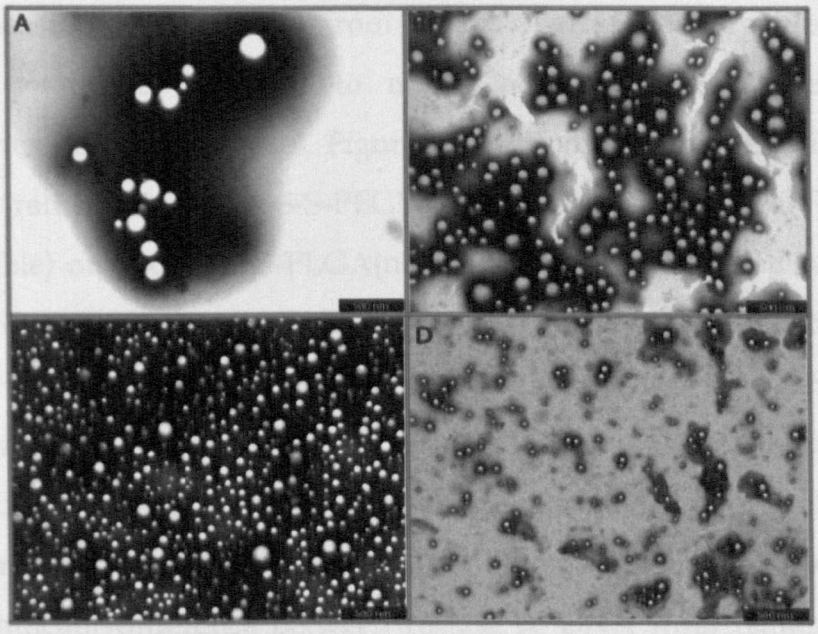


Figure 4-4. Transmission Electron Micrographs (TEM) of different (wt/wt%) ratio of PLGA-S-S-PEGMA$_{475}$-folate to PLGA-S-S-PLGA a) 10% wt , b) 25% wt , c) 50% wt and d) 75 % wt.

4.6.1. Encapsulation of plasmid DNA

The combination of the 'homo-diblock' PLGA-S-S-PLGA and the amphiphilic diblock PLGA-S-S-PEGMA$_{475}$ polymers with the double emulsion method resulted in efficient encapsulation of plasmid DNA (GWiz luciferase). The extent of incorporation was 80 % of the DNA in solution, corresponding to a theoretical loading of 0.4% with respect to the mass of polymer in the emulsion (~ 4ug DNA per mg of polymer). Recovery of DNA from the nanoparticles following treatment of the dried copolymers with 0.5% N NaOH indicated that 3.2ug/mg of plasmid DNA had been encapsulated as determined by UV spectroscopy and comparison to standard absorption values at 260 nm (Figure 4-21).

4.6.2. In vitro release profiles of plasmid DNA from nanoparticles

The release profiles of DNA from the nanoparticles were investigated under conditions designed to mimic reducing and non-reducing environments in the cytosol, Figure 4-5. Co-polymer particles in 50:50 molar ratios of PLGA-S-S-PEGMA$_{475}$ to either PLGA-S-S-PLGA (reducible) or PLGA-TEG-PLGA(non-reducible) were loaded with the same amounts of plasmid DNA (3.2mg.mg^{-1} polymer) and incubated in PBS pH 7.4 in the presence or absence of 5 mM glutathione (GSH). As apparent from Figure 4-5, the disulfide core- based nanoparticles showed 85% release of DNA over 16 hours in presence of 5mM GSH compared to 40% release in PBS buffer alone. Furthermore, there was no significant difference between release of DNA from nanoparticles containing the non-reducible PLGA-TEG-PLGA core system in the presence and absence of 5mM GSH (45% and 46% release, respectively).

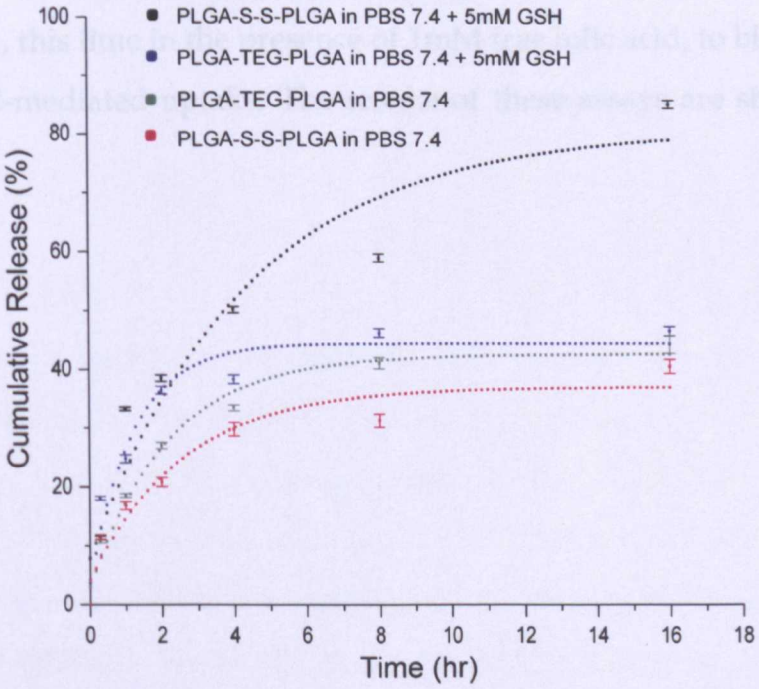


Figure 4-5. Cumulative releases (%) of plasmid DNA from nanoparticles.

4.6.3. Uptake of nanoparticles in Calu-3 cell line

The lung cancer cell line Calu-3 was chosen as a suitable model and clinically relevant in vitro test of nanoparticle efficacy. Recent studies have indicated that pemetrexed, a folate antimetabolite, is a potent and effective therapeutic in the treatment of lung cancers[40] a finding that suggests that folate-receptor (FR) mediated targeting[41] might be highly suitable for lung cancer cell lines. Accordingly, Calu-3 monolayers were grown and incubated with nanoparticle suspensions when the cells were confluent and linked via tight junctions as shown by trans-epithelial electrical resistance (TEER) measurements. The nanoparticles in the uptake assay contained progressively increased molar ratios of PLGA-S-S-PEGMA$_{475}$-folate relative to the PLGA-S-S-PLGA and PLGA-TEG-PLGA 'cores', while 'non-functional' PLGA-S-S-PEGMA$_{475}$-OH:PLGA-S-S-PLGA nanoparticles were used as a control for the PEGMA-folate surface. As a positive control, a second set of assays was carried out, this time in the presence of 1mM free folic acid, to block any specific FR-mediated uptake. The results of these assays are shown in Figure 4-6.

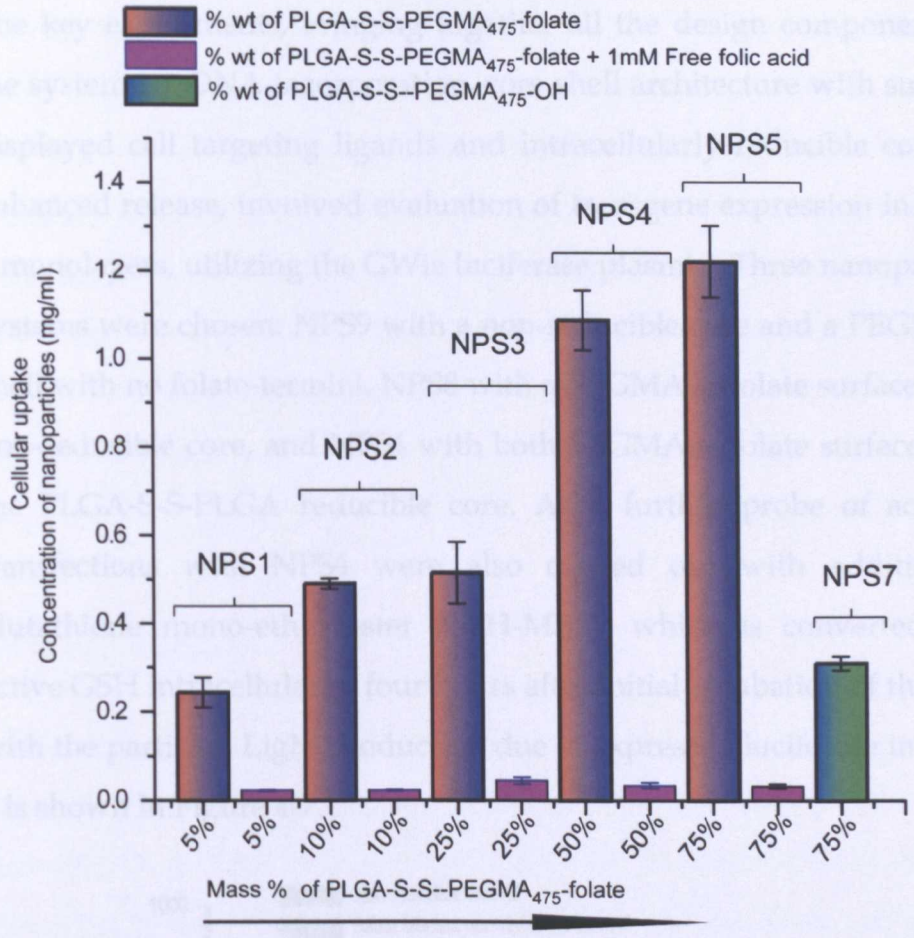


Figure 4-6. Folate mediated cellular uptake of nanoparticles by Calu-3 cell lines.

Cell uptake of nanoparticles increased with increased amounts of the folate-tipped block co-polymers in the matrix, although there was only a marginal difference between uptakes of the 75 % folate-tipped particles (NPS5) compared to 50 % (NPS4). Uptake of the hydroxyl-terminated particles (NPS9) was significantly lower ($p = 0.002$) than the folate-terminated systems. In all cases, addition of free folic acid suppressed uptake, consistent with a folate-receptor mediated uptake mechanism, rather than that due to the small variations (+/- 10 %) in particle size over the nanoparticle samples.

The key experiments, bringing together all the design components of the system i.e. DNA incorporation, core-shell architecture with surface-displayed cell targeting ligands and intracellularly reducible core for enhanced release, involved evaluation of transgene expression in Calu-3 monolayers, utilizing the GWiz luciferase plasmid. Three nanoparticle systems were chosen: NPS9 with a non-reducible core and a $PEGMA_{475}$ shell with no folate-termini, NPS8 with a $PEGMA_{475}$-folate surface but a non-reducible core, and NPS4 with both $PEGMA_{475}$ folate surfaces and the PLGA-S-S-PLGA reducible core. As a further probe of activity, transfections with NPS4 were also carried out with addition of glutathione mono-ethyl ester (GSH-MEE), which is converted into active GSH intracellularly, four hours after initial incubation of the cells with the particles. Light production due to expressed luciferase in Calu-3 is shown in Figure 4-7.

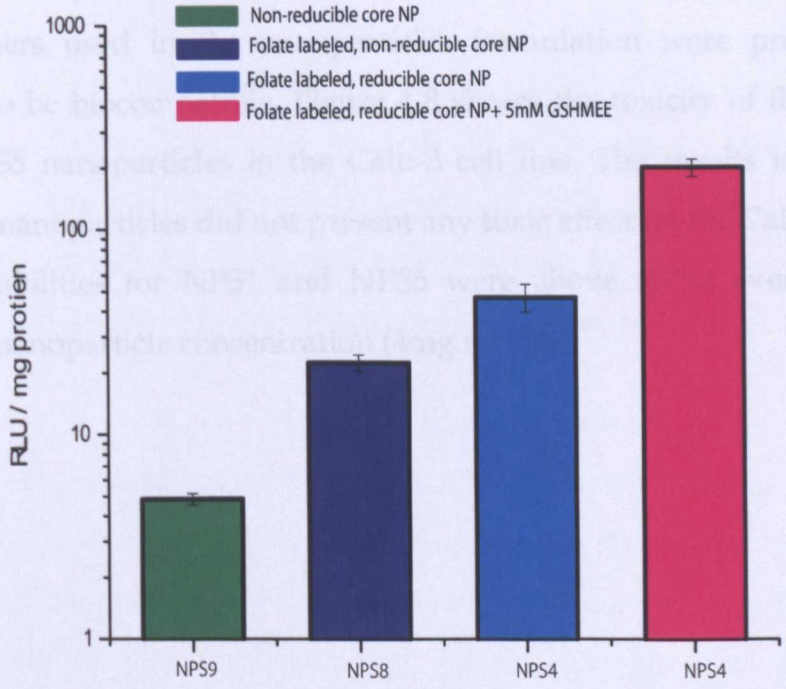


Figure 4-7. Transfection assays in Calu-3 monolayers with nanoparticles with/without cell-targeting and bioreducible functionality. RLU = relative light units, a measure of luciferase expression.

Overall transgene expression was higher for the bioreducible and folate-targeted nanoparticles, with the highest light production for cells incubated with NP4 with addition of GSH-MEE. Transfection increased 5-fold for the folate-functional NPs compared to the non-targeted non-reducible NPs, and 10-fold by incorporation of the additional reducible component in the cores. Boosting the intracellular GSH resulted in an overall increase of ~ 4-fold, indicating the importance of the bioreducible core.

4.6.4. Cell viability

MTS assay was chosen to determine the cytotoxicity of the nanoparticles on Calu-3 cells. Low toxicity of the nanoparticles was expected since the PLGA-S-S-PLGA and PLGA-S-S-PEGMA-folate copolymers used in the nanoparticles formulation were previously shown to be biocompatible. Figure 4-8 shows the toxicity of the NPS1 and NPS5 nanoparticles in the Calu-3 cell line. The results indicated that the nanoparticles did not present any toxic effect on the Calu-3 cells since viabilities for NPS1 and NPS5 were above (87%) even at the highest nanoparticle concentration ($4mg.mL^{-1}$).

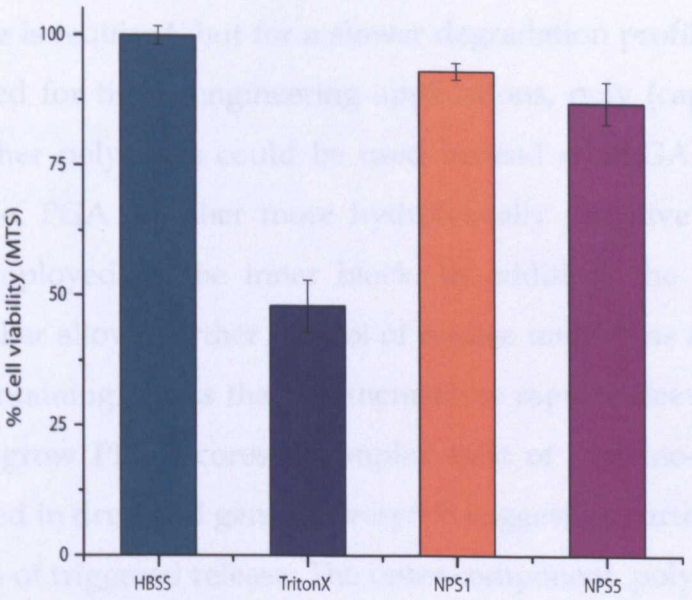


Figure 4-8. Cytotoxicity of NPS1 and NPS5 were compared to TritonX (positive control) and HBSS (negative control). Viability levels are represented as a percentage with respect to the level obtained for the negative control.

Taken together, the results of the uptake assays in the absence and presence of free folic acid and/or 'non-functional' OH-terminal particles, combined with the enhancements observed for transgene expression using folate-functional particles with bioreducible disulfide cores, are strong indicators of the success of the design strategy.

Overall goal was to establish the chemistries necessary for incorporating a wide range of potential biotherapeutic agents, such as proteins and nucleic acids, inside protective drug delivery vehicles that should be biocompatible, easily functionalisable for cell targeting, and bio-responsive. An important part of the strategy was that the components are in effect, modular, so different parts could be combined together or replaced, dependent on a particular desired application. The PLGA core was chosen because this polymer has been extensively used clinically, and the degradation rate can be tuned by co-monomer content and degree of crystallinity[42]. For gene delivery applications,

rapid release is required, but for a slower degradation profile as might be considered for tissue engineering applications, poly (caprolactone) (PCL) or other polyesters could be used instead of PLGA. For more rapid release, PGA or other more hydrolytically sensitive polyesters could be employed as the inner block. In addition, the use of the disulfide linker allows further control of release rate[43, 44] as a variety of disulfide containing blocks that are themselves rapidly cleavable could be used to grow PLGA cores. Examples exist of cysteine-terminated peptides used in drug and gene delivery[45,46] suggesting further possible mechanisms of triggered release. The outer component, poly (PEGMA), enables yet more flexibility in delivery vehicle design. $PEGMA_{475}$ homopolymer grown by ATRP was utilised, but co-polymers with other PEG-methacrylates offer the possibilities of tumour targeting through a thermally-responsive phase transition[28,29], while additional functionality than folate is readily accessible through the wealth of 'click' chemistries reported in the literature[47]. Recent data has indicated biocompatibility of PEGMA-based polymers grown by controlled radical routes[30] providing catalyst residues are effectively removed,[31] and thus it is likely that a platform of therapeutic carrier materials could be produced by this route.

Finally, the use of the double emulsion method allows blending of different polymers to tune degree of core-vs-shell components, and to encapsulate a variety of potent yet delicate biomacromolecules with high efficiency.

4.6.5. Conclusions

Modular block co-polymers were assembled via ROP, ATRP and click chemistry routes to generate biocompatible, cell-targeted and bioresponsive gene delivery vehicles. Block copolymers of PLGA-S-S-PLGA and PLGA-S-S-$PEGMA_{475}$ were synthesized using the above polymerisation techniques. These two polymers are connected by disulfide bridges to afford reduction in the presence of reducing agents. The terminal of the hydrophilic block, in this case $PEGMA_{475}$ has been used to install folate for receptor targeting. Double emulsion techniques were used to encapsulate DNA with high efficiency, and uptake of the resultant nanoparticles was shown to take place in Calu-3 cells via a specific folate-receptor mechanism. The reporter gene expression studies demonstrated that selective receptor-mediated and intracellularly triggered drug delivery took place, and the ability to do this in the clinically important Calu-3 cell line offers promise for future therapeutic applications.

4.7. Supplementary Figures (Figure 4-9 to 4-24)

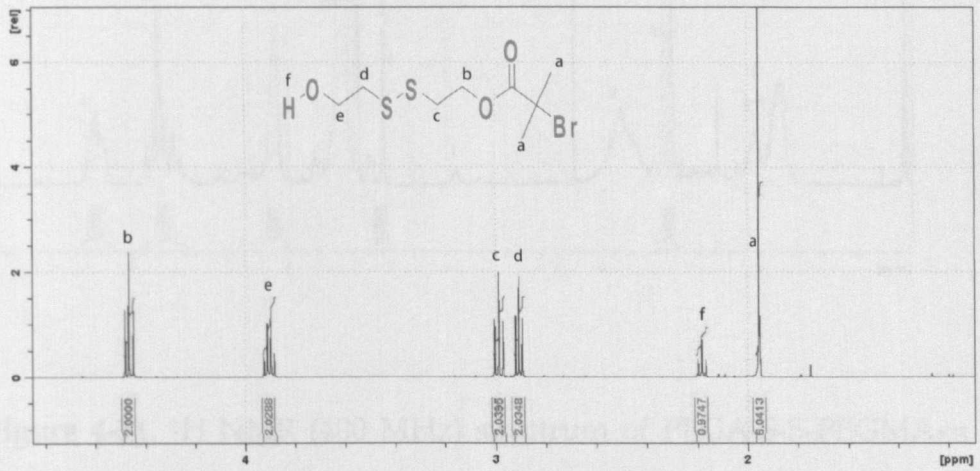


Figure 4-9. ^{1}H NMR (400 MHz) spectrum of 2-2((2-hydroxyethyl) disulfanyl) ethyl 2- bromo-2methylpropanoate in $CDCl_3$. Note: the OH signal at 2.2 ppm was not always visible, the presence of this signal was found to be dependent on the sample concentration.

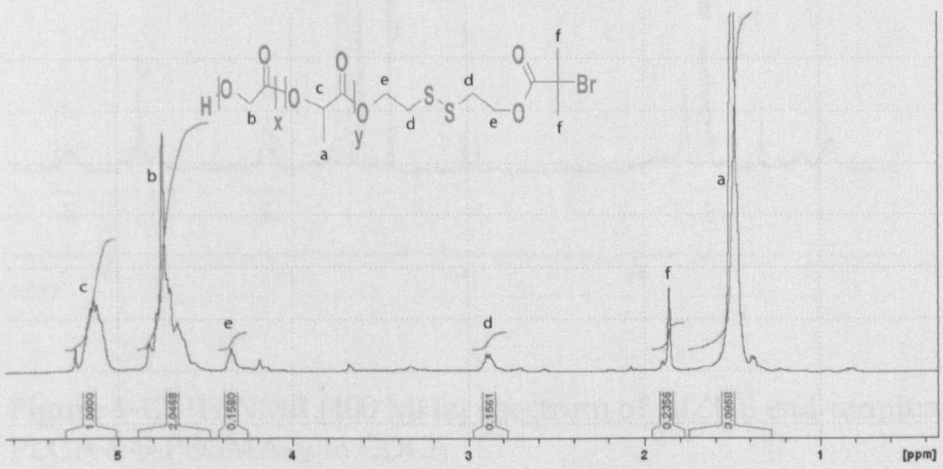


Figure 4-10. ^{1}H NMR (400 MHz) spectrum of PLGA-S-S-Br. in $CDCl_3$.

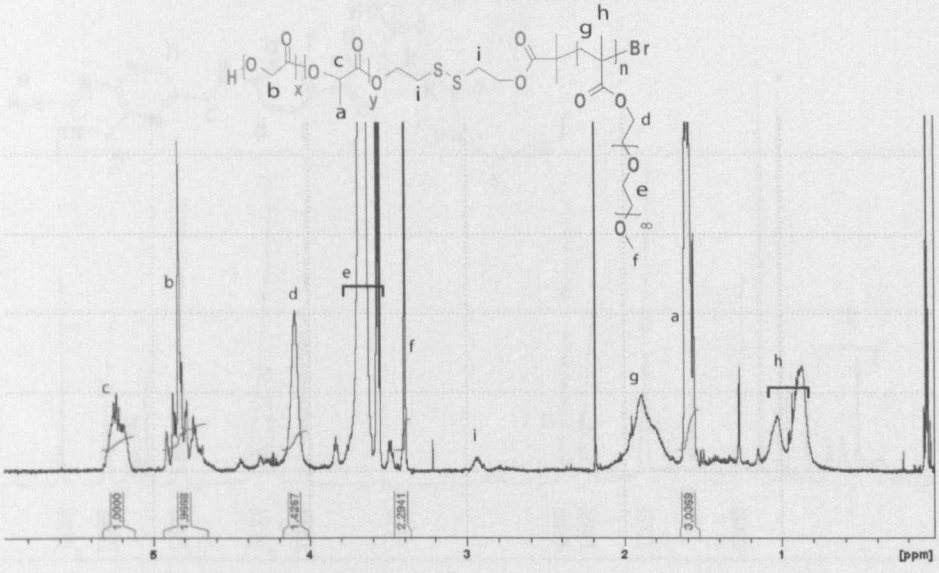


Figure 4-11. ^{1}H NMR (400 MHz) spectrum of PLGA-S-S-PEGMA$_{475}$ in $CDCl_3$.

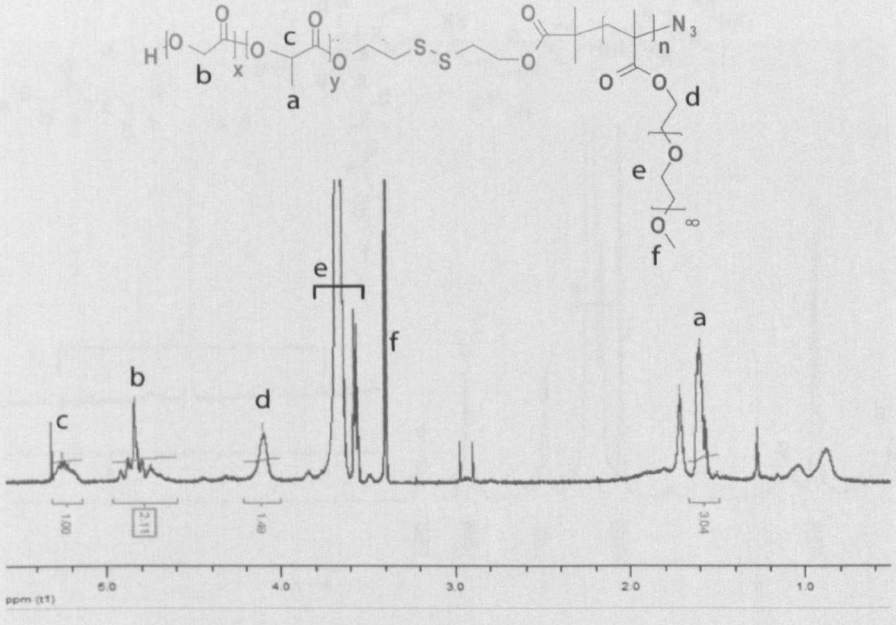


Figure 4-12. ^{1}H NMR (400 MHz) spectrum of AIZDE end-terminal PLGA-S-S-PEGMA$_{475}$ in $CDCl_3$.

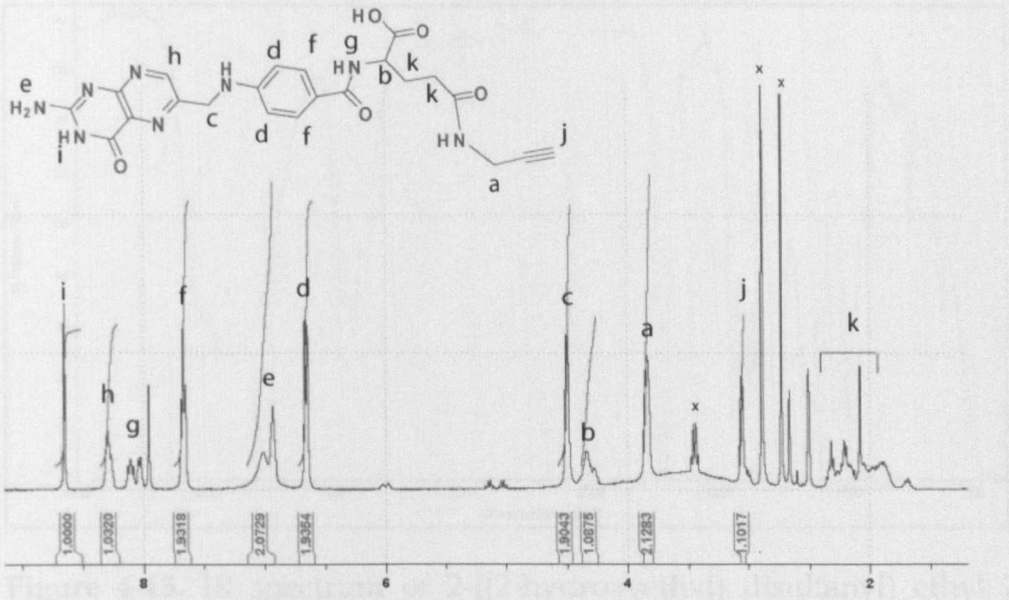


Figure 4-13. ^{1}H NMR (400 MHz) spectrum of acetylene folate in DMSO-d6.

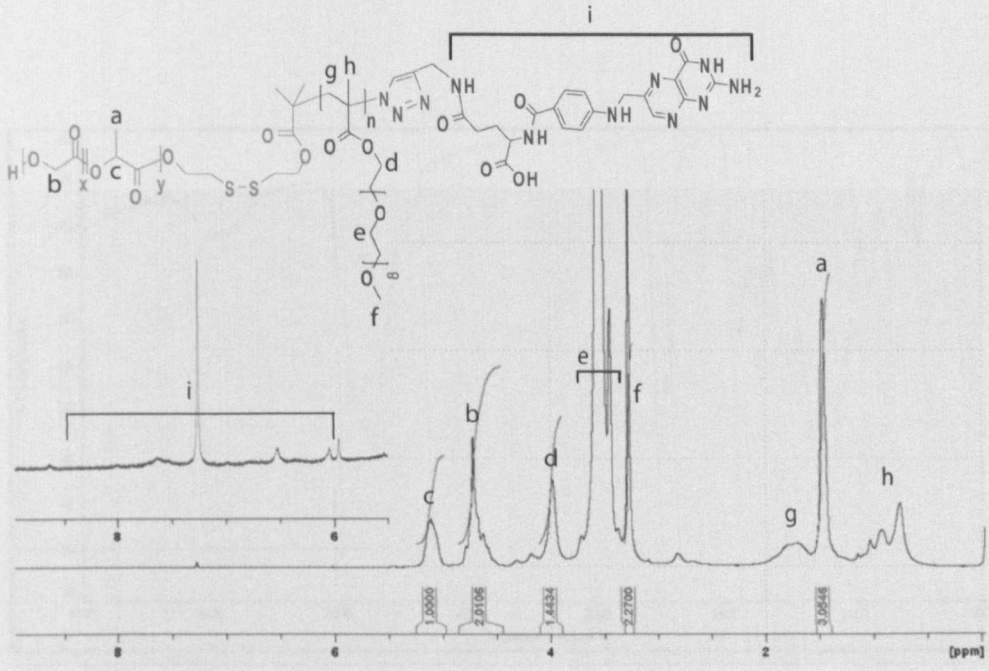


Figure 4-14. ^{1}H NMR (400 MHz) spectrum of PLGA-S-S-PEGMA$_{475}$-folate in $CDCl_3$.

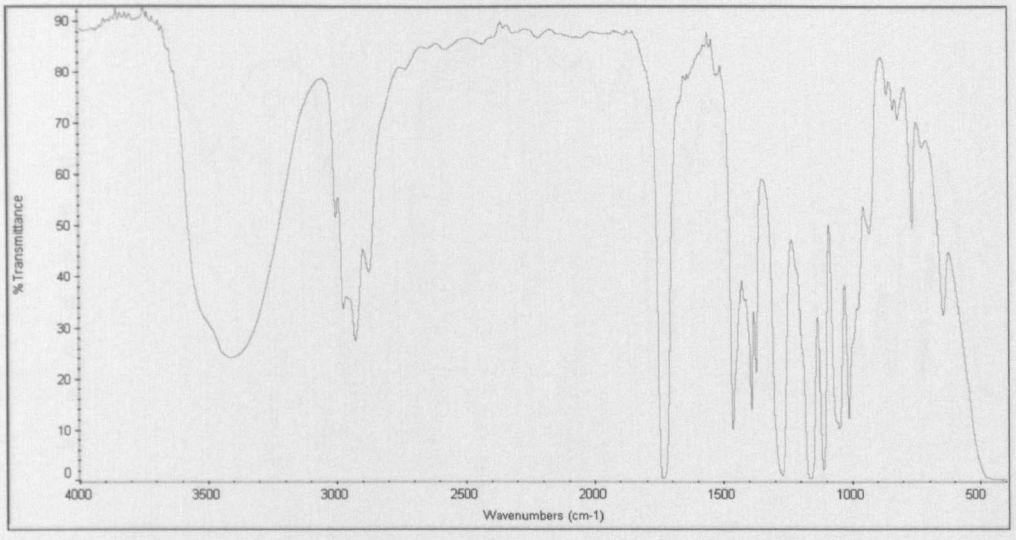


Figure 4-15. IR spectrum of 2-((2-hydroxyethyl) disulfanyl) ethyl 2-bromopropanoate, FTIR (cm^{-1}) 3410, (-OH), 2927, (C-H), 1734, (C=O).

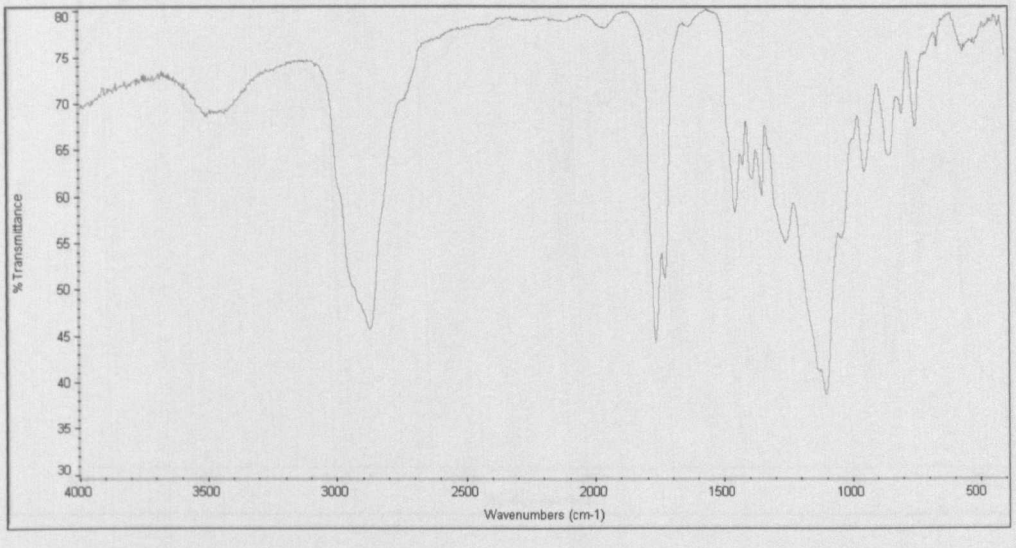


Figure 4-16. IR spectrum of PLGA-S-S-Br, FTIR (cm^{-1}) 2860–2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

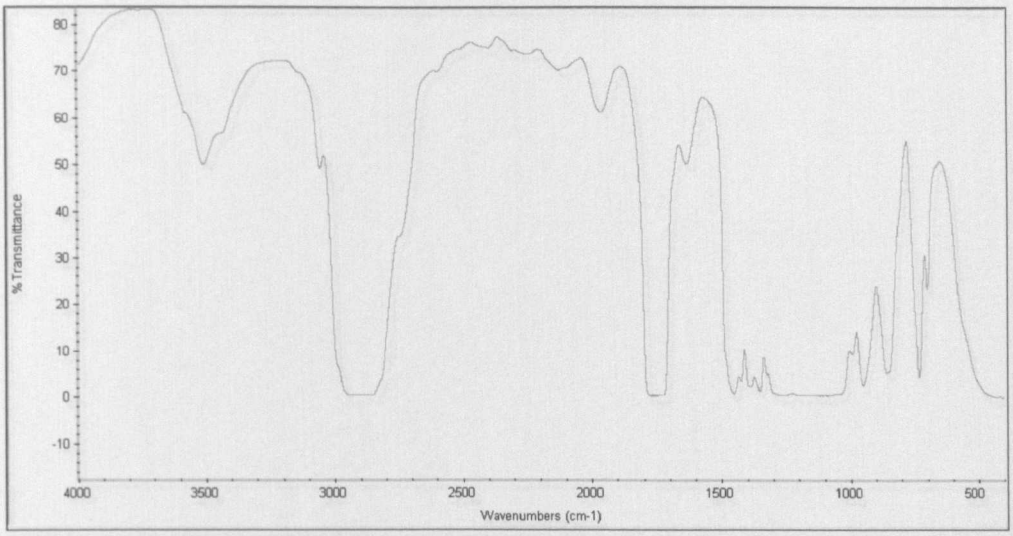


Figure 4-17. IR spectrum of PLGA-S-S-PEGMA$_{475}$, FTIR (cm^{-1}) 2860–2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

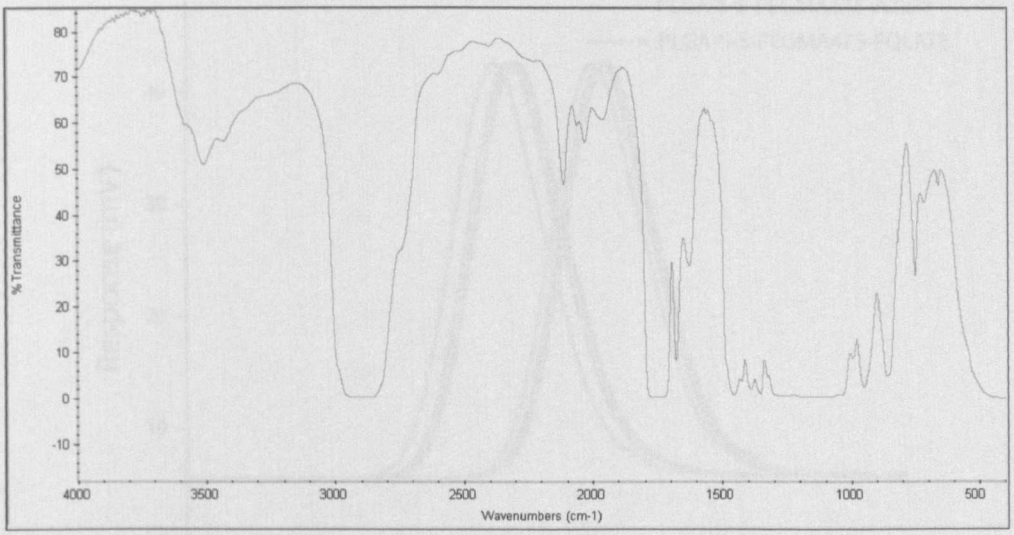


Figure 4-18. IR spectrum of AIZDE end-terminal PLGA-S-S-PEGMA$_{475}$, FTIR (cm^{-1}) 2860–2940 (C-H), 2106 (N3), 1760 (C=O).

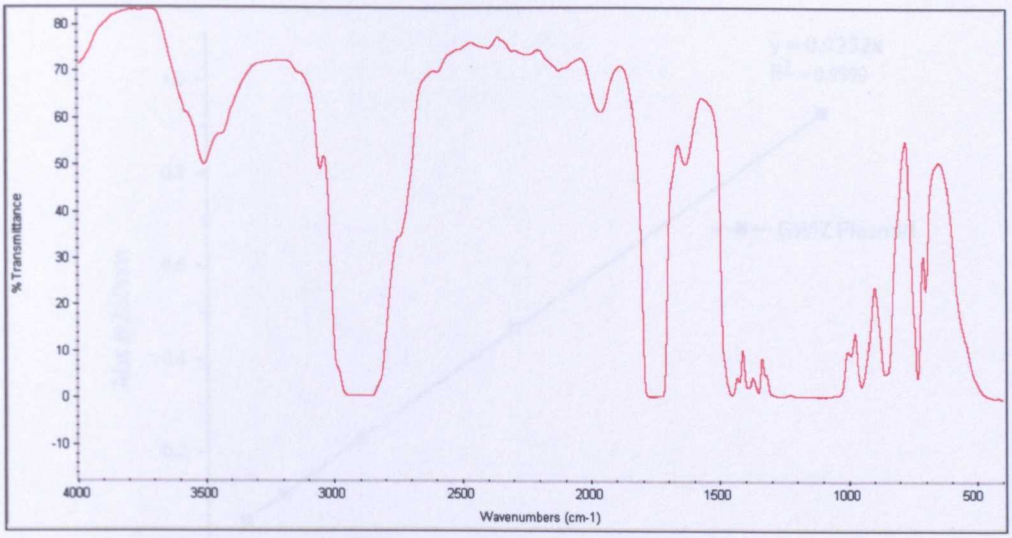


Figure 4-19. IR spectrum of PLGA-S-S-PEGMA$_{475}$-folate, FTIR (cm^{-1}) 2860–2940 cm^{-1} (C-H), 1760 cm^{-1} (C=O).

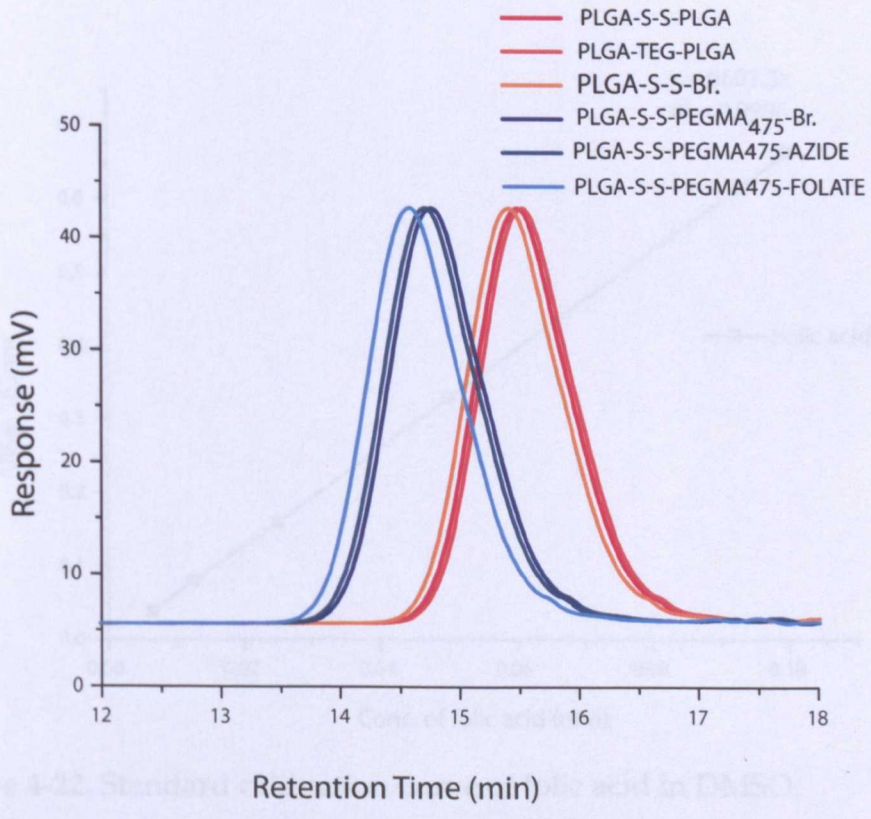


Figure 4-20. GPC chromatogram for polymer and diblock copolymers in chloroform eluent.

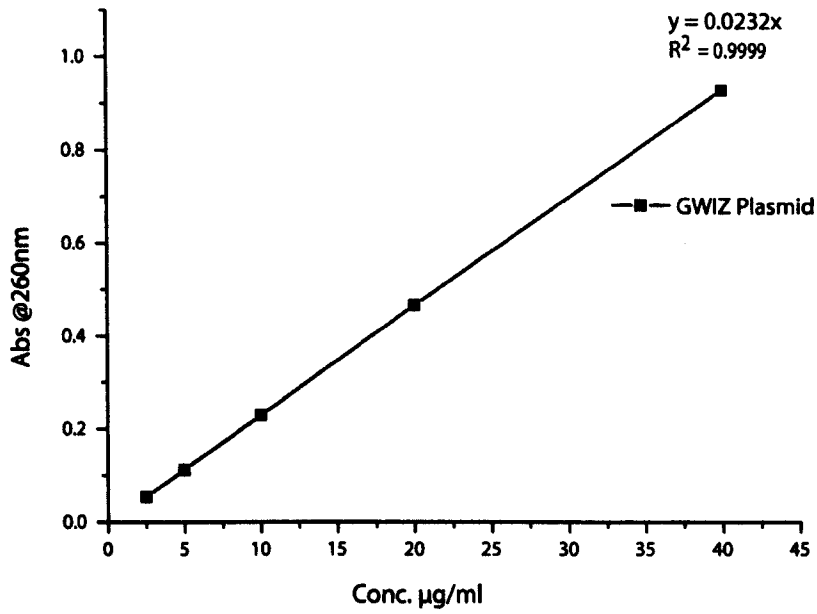


Figure 4-21. Standard calibration curve of GWIZ luciferase Plasmid in water.

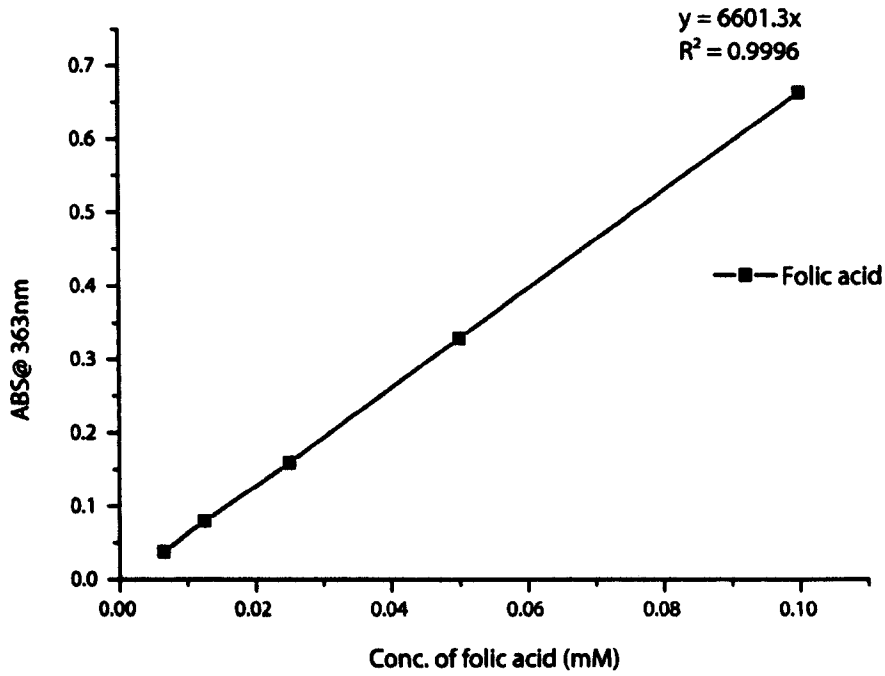


Figure 4-22. Standard calibration curve of folic acid in DMSO.

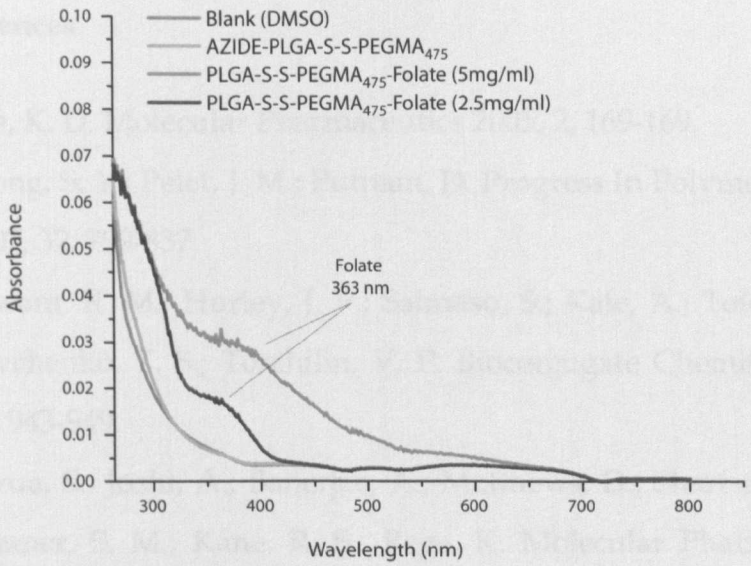


Figure 4-23. UV–vis spectra of the two different concentration of PLGA-S-S-PEGMA475-folate compared to AZIDE terminal PLGA-S-S-$PEGMA_{475}$.

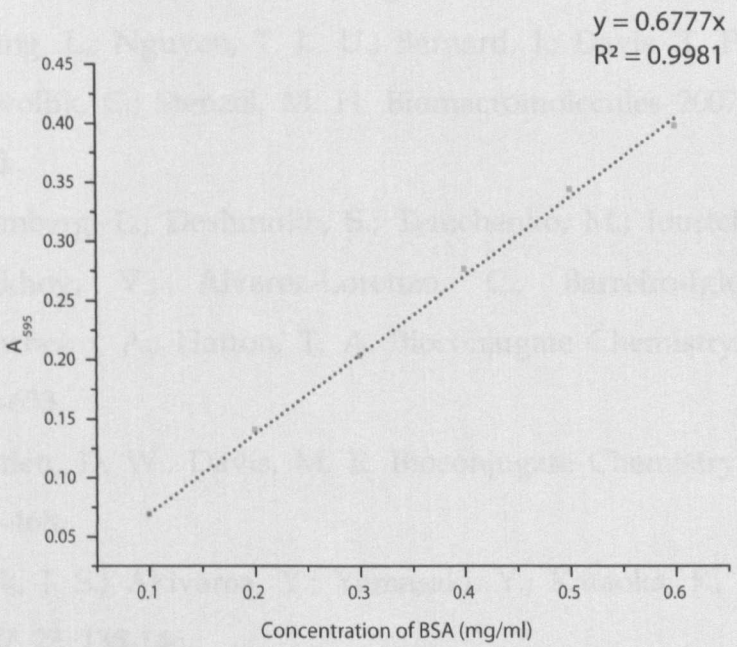


Figure 4-24. Standard calibration curve of BSA.

4.8. References

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Chapter 5

Final Conclusion

5.1. From Plain Nanoparticles to the Design of an Advanced Multifunctional Nanoparticle "Platform" as a Drug and Gene Delivery System

Despite intensive research and numerous clinical trials over the last two decades, successful results for gene therapies in humans to treat both acquired and inherited diseases remains elusive. The approach of using viral vectors to express genes for therapeutic intentions still suffers from cytotoxicity and immunogencity problems[1, 2]. This is highlighted by the recent (July 2007) death of a participant in a gene therapy trial a few days after the intra-articular injection of an adeno-associated viruses AAV vector[3]. In contrast, nonviral vectors have merit due to their low immunogenicity. Thus, research on the designing and synthesis of nonviral based gene delivery systems has accelerated to replace viral vectors. However, the efficacy of nonviral delivery methods is still far below that of viral vectors[4].

The existence of many extracelullar and intracellular barries limits the transport and biodistribution abilities (pharmacokinetics) of nonviral gene delivery systems resulting in low transfection efficiency. In addition to these barriers, there are other factors that have an effect on the overall efficacy of delivery systems which are directly related to the intrinsic properties of the carrier systems, these include physical properties (size, surface charges), loading efficiency

of DNA, release profiles and presense of specific targeting moities to ensure localistion of the delivery carriers where they are needed.

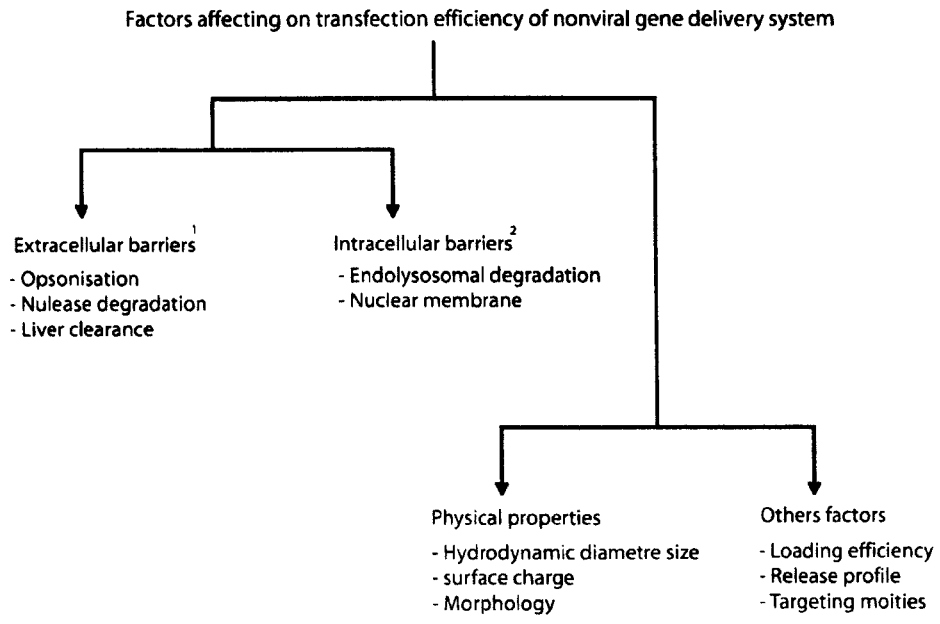

Figure 5-1. Factors affecting transfection efficiency of nonviral gene delivery systems.

The main current challenges in gene therapy are the design of more sophisticated nonviral gene delivery systems that are able to circumvent physiological barriers. In addition, controlling the physicochemical properties of the carrier (e.g. size, surface charges and morphology) and formulation parameters (e.g. loading efficiency, release profile and specific targeting) are considered to be crucial in determining the overall transfection efficiency of nonviral gene delivery vectors. Moreover, the long term safety of these carriers for *in vivo* applications requires formulations which are composed of biodegradable and biocompatible materials.

Therefore, combining all these properties that are required for the development of an ideal nonviral gene delivery system in a single

vector remains a difficult task and has been focus for many groups over the last few years.

5.2. Enhancing encapsulation efficiency of DNA in PLGA nanoparticles

Synthetic copolymers of poly(lactic-co-glycolic acid) PLGA are the most widely studied polymer-based delivery system due to their demonstrated FDA-approved track record as a vehicle for drug and protein delivery. Although PLGA based delivery systems are safe and useful for protein delivery, their application for delivery of plasmid DNA suffers several drawbacks.

Drawbacks of PLGA based carrier for plasmid DNA delivery:

1- Low encapsulation efficiency; owing to the large size and hydrophilic nature of DNA, the encapsulation of nucleic acids in hydrophobic PLGA particles is a challenge.
2- Degradation of DNA during formulation of nanoparticles; double emulsion techniques were commonly used to encapsulate pDNA in the matrix of particles and this requires high shear forces (e.g. homogenisation and sonication) to reduce the particle size to micro- and nanoscale range.
3- Hydrolytic degradation of PLGA polymer generates an acidic climate with a pH as low as 1.5 inside the nanoparticle matrix and potentially results in degradation of DNA and compromising of biological activity.
4- The rate of pDNA release is often too slow, this may be due to unwanted interaction between DNA and PLGA polymer and thus in case of DNA vaccination, this may prevent an optimal immune response.

5- Plain PLGA based delivery systems lack the targeting specificity and have short circulation times owing to the absence of a protective hydrophilic shell or targeting ligands.

Therefore, given the limitations with PLGA based delivery systems, further developments and modifications are needed to obtain a carrier that fulfils the requirements of an ideal gene delivery system.

Several approaches have been developed already to improve the PLGA delivery system such as encapsulation of excipients into PLGA micro and nanoparticles to buffer the acid microenvironment so as to protect the DNA from degradation. Cationic polymers were used to condense DNA before encapsulation in PLGA matrix to enhance encapsulation and also to protect DNA from degradation through buffering the acidic microenvironment (through protonation of amine groups) generated inside the PLGA particles[5]. A similar approach, using poly (ethyleneimine), or chitosan has been reported for loading PLA particles with DNA[6]. Although these modifications have shown some success, there is concern over the potential cytotoxicity of some of the excipients.

These previous works have given an insight into the limitations of PLGA-based delivery systems and some current approaches which have been used to optimise the efficiency of the system. In addition to that, whilst modifying the PLGA based delivery system, it is very important to keep a good safety profile by using excipients which are also biocompatible or biodegradable.

Therefore, the next step adopted in this thesis was to use biocompatible excipients with PLGA based systems in order to overcome the limitations which are mentioned earlier. Csaba *et al,*

have explored a new nanocarrier system composed of PLGA polymer blended with PEO derivatives (poloxamers and poloxamines) to enhance the encapsulation efficiency of pDNA as well as improve release profile, details of this work have been discussed in Section 2 of Chapter 2. Although this work offered a versatile technique with very mild conditions to encapsulate pDNA and also provided a continuous release profile, the encapsulation efficiencies of pDNA were only achieved up to 35% - 45%. This has created a gap to introduce other excipients which could possibly improve the release profile of pDNA as well as increase the encapsulation efficiencies while keeping the good safety profile of PLGA based system.

5.3. PLGA : Jeffamine blended nanoparticles as DNA delivery system

The initial aims of this thesis were set on the development of blend nanoparticles based on PLGA polymer and Jeffamine™ (Poly (ethylene glycol) bis (3-aminopropyl) terminated. The rationale for choosing and adding Jeffamine™ to PLGA as excipient were mainly to obtain a higher encapsulation efficiency compared to available systems and also to improve release profile of DNA. The formation of these mixed systems is based on the phenomenon that PLGA polymer and Jeffamine are readily miscible via hydrogen bonding. Moreover, Jeffamine polymers have PEO backbones with two accessible amine groups at the ends of each polymer chain.

Therefore, the initial thoughts were to investigate the effect of blending Jeffamine with PLGA polymer on encapsulation efficiency of cDNA and releasing profile. Jeffamine polymers have the following characteristics:

1- Can bind with cDNA via hydrogen bonding as well as electrostatic interaction between negative phosphate groups on the cDNA (calf thymus DNA) backbone and positively charged amine groups (protonation of the terminal amine groups).
2- Upon protonation of terminal amine groups on Jeffamine polymer, they may play a role in buffering the acidic microenvironment which generates from hydrolytic degradation of PLGA polymers.
3- The presence of Jeffamine within the matrix of PLGA particles can improve the release profile via formation of hydrophilic channels upon erosion of the PLGA matrix.

A similar double emulsion techniques developed by Csaba *et al* were used for work in this thesis; it offers mild conditions during encapsulation of DNA in the matrix.

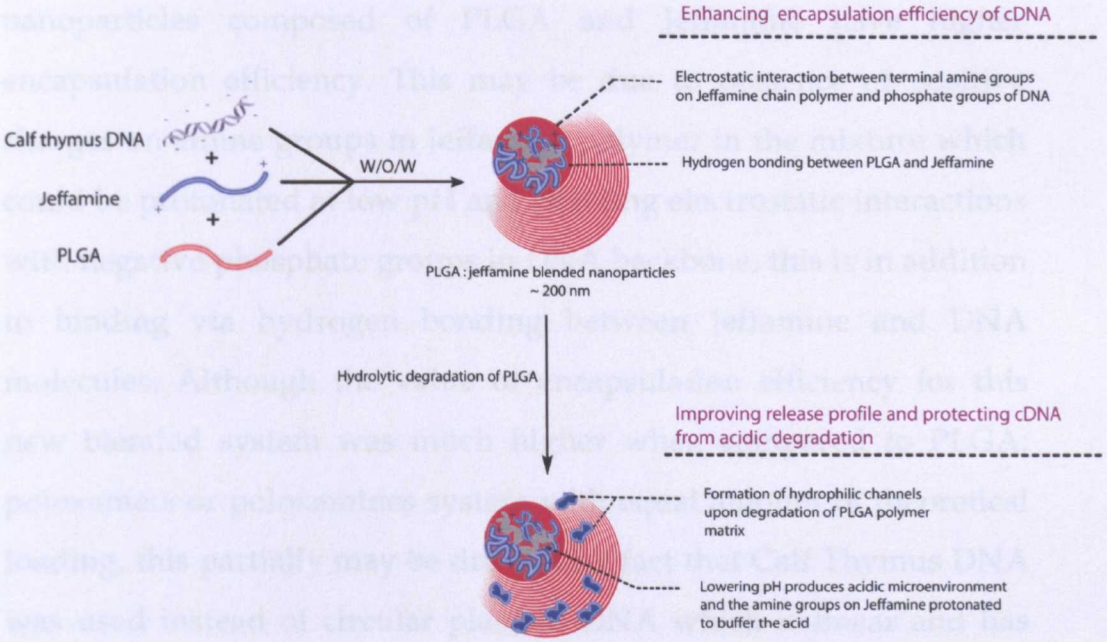

Figure 5-2. Schematic presentation of PLGA: Jeffamine nanoparticles system

$$E.E.(\%) = \frac{\text{Theoretical loading of DNA - un-encapsulated DNA}}{\text{Theoretical loading of DNA}} \times 100$$

From the comparisons of percentage of encapsulation efficiencies for different PLGA: excipient blended system, it appears that nanoparticles composed of PLGA and Jeffamine have higher encapsulation efficiency. This may be due to presence of positive charges on amine groups in Jeffamine polymer in the mixture which could be protonated at low pH and enabling electrostatic interactions with negative phosphate groups in DNA backbone, this is in addition to binding via hydrogen bonding between Jeffamine and DNA molecules. Although the value of encapsulation efficiency for this new blended system was much higher when compared to PLGA: poloxamers or poloxamines system with equal amount of theoretical loading, this partially may be due to that fact that Calf Thymus DNA was used instead of circular plasmid DNA which is linear and has smaller size. Nonetheless, these new blend system of PLGA and Jeffamine offer potential advantage for DNA delivery.

Generally, the release of DNA from PLGA nanoparticles follows a tri-phasic pattern[7]. There is an initial burst release of DNA molecules that are loosely associated with the surface of the particles and entrapped during solvent diffusion process of the double emulsion technique. The second is characterised by a slow erosion of the PLGA matrix. In the final phase, the DNA molecules start to release in a more controlled manner through the formation of hydrophilic channels in the matrix. Therefore, it can be said that hydrolytic process of PLGA is important in determining the rate of release of DNA. Blending of hydrophilic polymers such as Jeffamine in the hydrophobic matrix can have a positive effect on the release of DNA

molecules. Therefore the effect of blending of Jeffamine has been investigated in this section of the thesis and the results were indicative of continuous release pattern. This is believed to be due to strong hydration of the PLGA matrix by the presence of Jeffamine as hydrophilic polymer. A similar result has been obtained by other groups by blending poloxamines and poloxamers within PLGA nanoparticles.

Despite the works that have been carried out in this section of the thesis to enhance encapsulation efficiency of DNA within hydrophobic PLGA nanoparticles as well as improve release profile, the PLGA: Jeffamine blended nanoparticles still lacked the hydrophilic protecting shell on the surface to prevent interaction between particles and with an *in vivo* environment. Uncoated particles can be easily recognised by mononuclear phagocyte system (MPS) and rapidly cleared from blood; this is attributed to the hydrophobic nature of the PLGA particles that favour the adsorption of blood components such as opsonins. Therefore, the next challenge was to coat the surface of developed PLGA: Jeffamine blended nanoparticles with hydrophilic polymers; this is discussed in next section.

5.4. Surface modifications of PLGA: Jeffamine blended nanoparticles

The conventional approaches for surface modification of PLGA nanoparticles consisted of simple physical adsorption of block copolymers[8]. Surface modifications of PLGA nanoparticles have been carried out using either adsorption via electrostatic interactions or adsorption via hydrophobic interaction. PLGA polymers that form the bulk matrix of the nanoparticles impart hydrophobic character to

the surface of nanoparticles; whereas, the exposed carboxylic groups on surface of PLGA nanoparticles produce considerable negative charges of about - 50 mV (zeta potential) when measured in low ionic strength solution of PBS at fixed pH value of 7.4. It should be mentioned that blending of Jeffamine with PLGA polymers led to a less negative surface charges of about - 30 mV and this still allowed for surface modification to carry out via electrostatic interaction.

In order to test the two approaches, pDMAEMA-PEGMA and PLGA-PEGMA block copolymers were obtained. In both cases PEGMA segments represented the hydrophilic blocks whereas, pDMAEMA and PLGA represented the cationic block and hydrophobic block respectively. It was possible to adsorb these two block copolymers on the surface of PLGA: Jeffamine blended nanoparticles since the hydrophobic PLGA blocks can anchor on the surface via hydrophobic interaction and pDMAEMA via electrostatic interactions. On the other hand, the hydrophilic PEGMA can protrude towards the aqueous medium and provide a protective shell through steric hindrance.

The surface adsorptions of these block copolymers were studied by measuring the zeta potential value before and after the adsorptions. In both cases, the zeta potential values changed to near zero, an indication of successful adsorption of the block copolymers on the surface of the nanoparticles.

Despite the success of the adsorption experiment, there are limitations to physical adsorption methods. Owing to nonspecific attachments, these block copolymers can be easily displaced by serum proteins.

This undesired displacement can ultimately compromise the stability of the nanoparticles in the circulation and expose the particles to

aggregation. To overcome this problem, the alternative approach to provide the particles with a protective shell would be the synthesis of block copolymers, in particular, via controlled living polymerisation technique which allows designing the copolymers with control over the molecular weight and functionality, (discussed in next section).

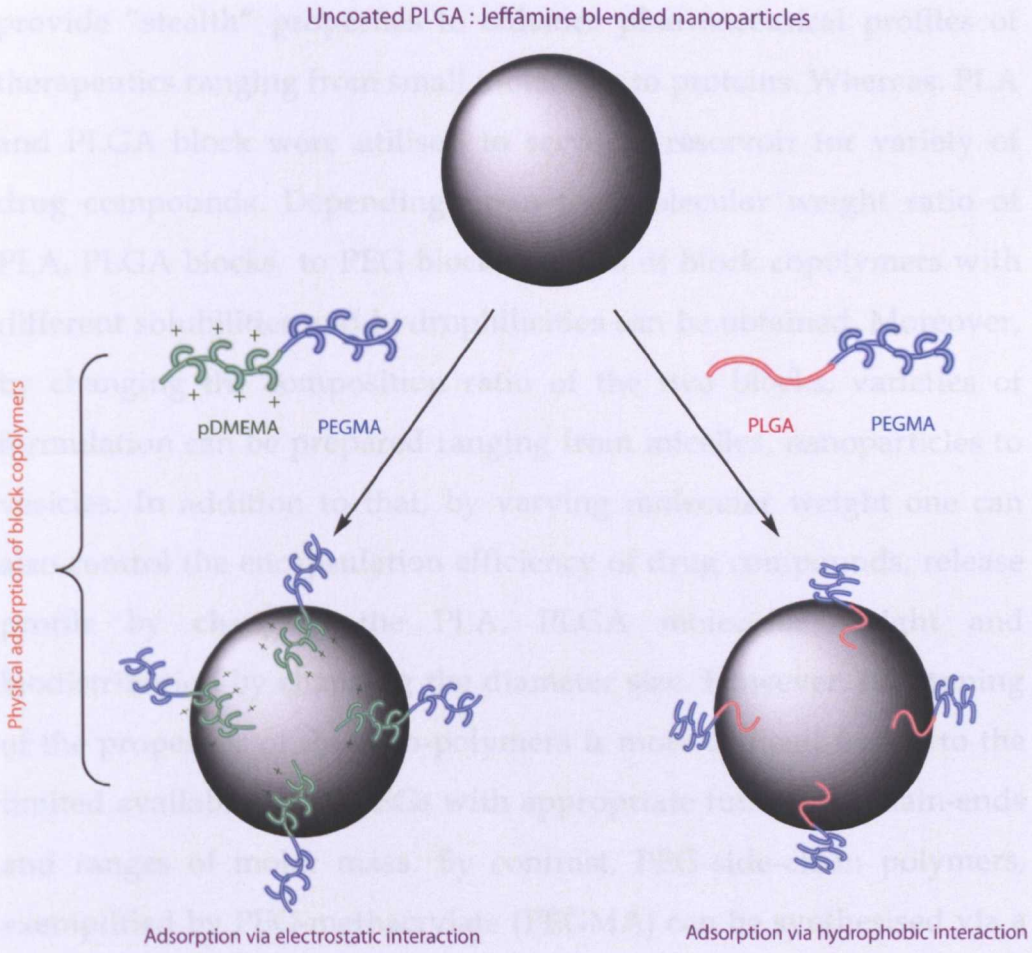

Figure 5-3. Schematic illustration of physical adsorption of pDMAEMA-PEGMA (left) and PLGA-PEGMA (right) on the surface of PLGA: Jeffamine blended nanoparticles.

5.5. Synthesis of biocompatible and degradable block copolymer

Conventionally, amphiphilic copolymers of PLA-PEG and PLGA-PEG were prepared for variety of purposes ranging from micelles, nanoparticles formulation or physically adsorbed on PLGA based nanoparticles to increase stability. The PEG block was used to provide "stealth" properties to enhance pharmaceutical profiles of therapeutics ranging from small molecules to proteins. Whereas, PLA and PLGA block were utilised to serve as reservoir for variety of drug compounds. Depending upon the molecular weight ratio of PLA, PLGA blocks to PEG block, a series of block copolymers with different solubilities and hydrophilicities can be obtained. Moreover, by changing the composition ratio of the two blocks, varieties of formulation can be prepared ranging from micelles, nanoparticles to vesicles. In addition to that, by varying molecular weight one can also control the encapsulation efficiency of drug compounds, release profile by changing the PLA, PLGA molecular weight and biodistribution by changing the diameter size. However, fine-tuning of the properties of these co-polymers is more difficult owing to the limited availabilities of PEGs with appropriate functional chain-ends and ranges of molar mass. By contrast, PEG-side-chain polymers, exemplified by PEG-methacrylate (PEGMA) can be synthesised via a variety of routes, and very fine manipulation of properties can be exerted through controlled polymerisation strategies. Controlled radical polymerisations, primarily ATRP and RAFT have enabled PEG-methacrylates and co-polymers with exciting new properties to be produced.

In Chapter 3 of the thesis, and for the first time, a series of PLA-PEGMA and PLGA-PEGMA copolymers were prepared via combining controlled living polymerisation and ring opening polymerisation techniques.

The main objectives of this work were to combine ring opening polymerisation (ROP) with Radical Addition Fragmentation Transfer (RAFT) technique to synthesise a series of PLA-PEGMA and PLGA-PEGMA block copolymers. These copolymers were varied in co-monomer content and block length with low polydispersities. Owing to the amphiphilic nature of these block copolymers, the materials can form micelles in aqueous solution. In contrast, it is possible to obtain nanoparticles by using emulsion techniques such as single or double emulsion techniques. This new synthesis approach offer significant advantages not only because these block copolymers are biocompatible and biodegradable, but also through fine control over the properties of the system. The tailoring of co-monomer type and the wide variety of methacrylate and acrylate functional materials available that can be polymerised via RAFT or ATRP means that fine-tuning of drug incorporation and release should be possible.

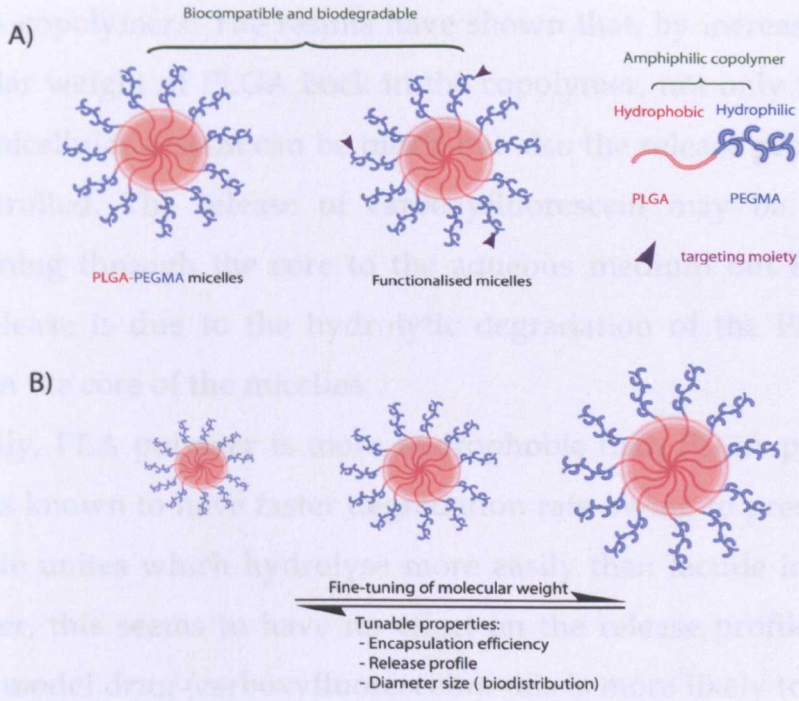

Figure 5-4. Schematic illustration of A) PLGA-PEGMA micelles and functionalised micelles. B) Tuning the properties of the micelles by changing the molecular weight.

The choice of RAFT agent was important to combine the two techniques, ROP and RAFT polymerisations. Therefore, BSTSE RAFT agent, which has an incorporated hydroxyl moeity on the R group, was chosen to initiate the ROP of lactide and glycolide monomers. The obtained PLA and PLGA polymers carrying the RAFT agent at the end of the chain were subsequently utilised as macro-initiator to grow the second polymer in a controlled manner. After synthesis of different molecular weights of PLA-PEGMA and PLGA-PEGMA, the next step was to investigate the effect of molecular weight on size, encapsulation efficiency and release profile. This was a crucial experiment to show that the developed synthetic system for this particular copolymers could be utilised to control the properties of the micelles system. A model drug (carboxyfluorescein) was encapsulated in the hydrophobic core of PLA-PEGMA and PLGA-

PEGMA copolymers. The results have shown that, by increasing the molecular weight of PLGA bock in the copolymer, not only the size of the micellular system can be tuned but also the release profile can be controlled. The release of carboxyfluorescein may be due to partitioning through the core to the aqueous medium but for long term release is due to the hydrolytic degradation of the PLA and PLGA in the core of the micelles.

Generally, PLA polymer is more hydrophobic than PLGA polymer. PLGA is known to have faster degradation rate owing to presence of glycolide unites which hydrolyse more easily than lactide in water. However, this seems to have no effect on the release profile of the chosen model drug (carboxyfluorescein), this is more likely to be due to the nature of this molecules which is not strongly hydrophobic. Therefore, its retention in the hydrophobic core of the micelles was not significantly affected by using PLA or PLGA polymer.

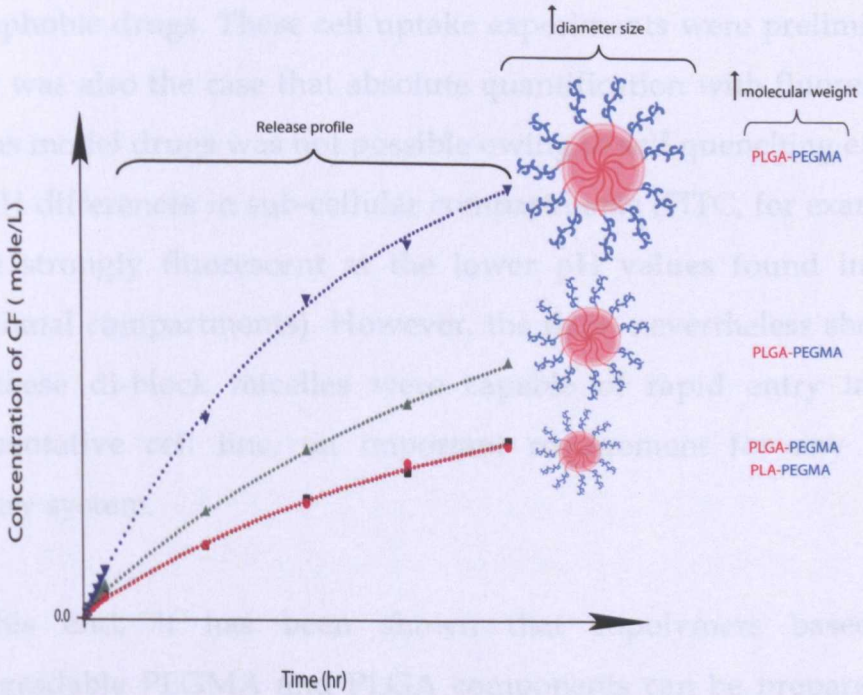

Figure 5-5. Schematic illustration of CF release profile varies by changing the molecular weight of the copolymer.

By comparing the new developed synthetic system to conventional techniques to prepare PLGA-PEG copolymers, in the new system it is more easily possible to alter model drug incorporation and release via changes in co-polymer structure. Although this was not fully explored in ths thesis, the enhanced control inherent to the synthesis of these PLGA-PEGMA block co-polymers by combined ROP and RAFT allows much better flexibility in co-polymer preparation than the anionic routes needed for PLA-PEG systems.

Cellular uptakes is an important requirement of any drug delivery system, therefore, the next stage was to examine the uptake of these micellar system by a representative cell lines for which 3T3 fibroblast cell line has been chosen.

Fluorescein isothiocyanate (FITC) was selected as complementary to CF as it is more hydrophobic and therefore a better model for more hydrophobic drugs. These cell uptake experiments were preliminary and it was also the case that absolute quantification with fluorescent dyes as model drugs was not possible owing to self-quenching effects and pH differences in sub-cellular compartments (FITC, for example, is not strongly fluorescent at the lower pH values found in late endosomal compartments). However, the data nevertheless showed that these di-block micelles were capable of rapid entry into a representative cell line, an important requirement for any drug delivery system.

To this end, it has been shown that copolymers based on biodegradable PEGMA and PLGA components can be prepared by combining ROP and RAFT chemistries with control over the molecular weights and polydispersity. The advantages of this

approach over the already existing methods to formulate drug and gene delivery systems have also been highlighted.

Despite the benefits that these materials have rendered to drug and gene delivery systems, some applications remain challenging; for instance, specific targeting to the action site or efficient drug/gene delivery inside the target cell. In this context, the design of multifunctional carrier systems could significantly improve already existing carrier characteristics and help to surmount these challenges. In case of nucleic acid delivery, the hurdles are much more complicated. DNA and RNA delivery vehicles are required to package the polynucleotide, protect it during transport in and across numerous biological environments and barriers, then release the biopolymer in the correct subcellular location. Therefore, the design and synthesis of more sophisticated carrier systems (multifunctional carriers) was the focus of Chapter 4, potentially for gene and drug delivery system.

5.6. Multifunctional Bioresponsive Cell-Targeted Block Co-polymer Nanoparticles for Gene Delivery

For DNA-based therapies the target is the cell nucleus, whereas for RNA, delivery to the cytoplasm is required. Many designed polymeric nucleic acid delivery systems (usually termed 'vectors') have been developed by analogy with viruses, which are, of course, natural DNA or RNA carriers that are able to deliver their payloads to cells with extreme efficacy. The requirements for these vectors are stringent: for example for DNA, the nucleic acid (which can be several million Da in molar mass) must be condensed to a small size or encapsulated, and protected from serum and intracellular nucleases. The DNA must be delivered to the target cell, cross the

external cell membranes passively or actively, leave the endosomal compartments (avoiding degradative enzymes and escaping traffic to the lysosomes), then it must translocate into the nuclear compartment ready for transcription. The vector must thus protect the DNA and help transport it across multiple cell barriers, yet release it at the correct time.

In addition, for an injectable DNA delivery formulation, the vectors with encapsulated/complexed DNA must be capable of extended circulation in the bloodstream in order to have chance to reach their cellular target. They must also be small enough to gain access to tissues and cells - typically this will put a size limit of <250 nm for the hydrodynamic diameter of the vector system in circulation. Above all, the vectors must be safe, i.e. non-toxic, non-immunogenic and fully cleared from the body after use. Block co-polymer micelles, vesicles and nanoparticles have been extensively investigated as carriers for nucleic acids, as the core-shell architectures enable packaging of DNA or RNA into a protected interior phase.

Designing and synthesis of a carrier system that can overcome all the challenges associated with DNA, RNA delivery pathways remain a difficult task to accomplish. Although there are many systems that have been already reported, but these systems do not have all the properties required for advanced gene delivery system in a single construct or they have been synthesised through many difficult chemistry steps. Moreover, one of the present limitations for the combination of multiple functions on a single particle is the surface chemistry required. Most of the developed systems rely on non-covalent strategies, such as electrostatic and biospecific interactions or hydrophobic adsorptions, which do not enable control over the composition, size and multifunctionality of the nanoparticulate system.

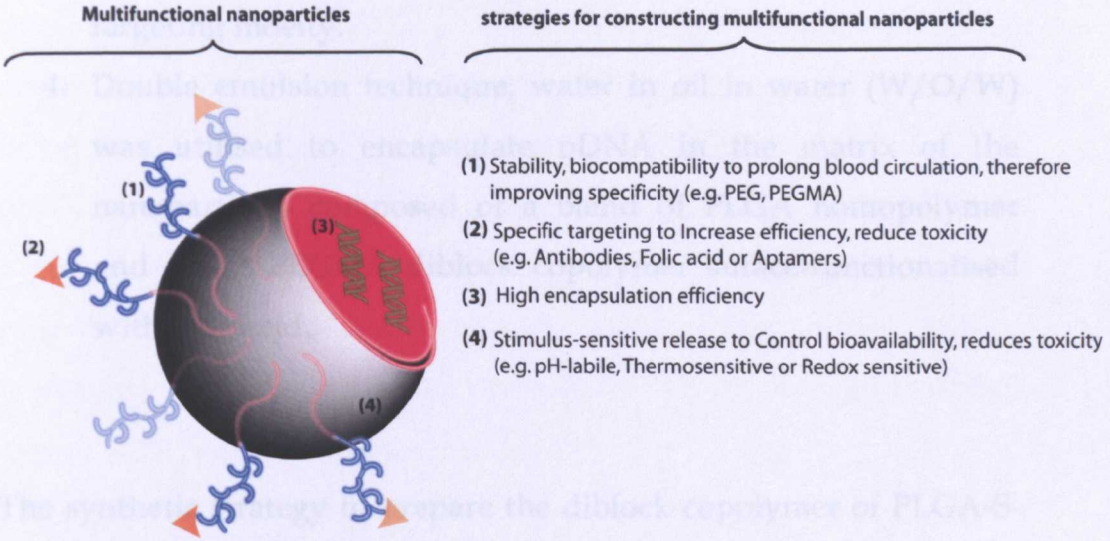

Figure 5-6. Schematic illustration of multifunctional nanoparticles for drug and gene delivery.

To build multifunctional nanoparticles that include all the above listed properties (Figure 5-5) in a single construct still represents a challenge but it is a goal that is becoming increasingly realistic and was the main focus of Chapter 4. In order to fabricate the multifunctional nanoparticles, the following strategies have been implemented:

1- Block copolymers of PLGA (the hydrophobic core block) and $PEGMA_{475}$ (the hydrophilic outer block) were synthesised using ring opening polymerisation (ROP) and Atom Transfer Radical Polymerisation (ATRP), these two blocks connected by bioreducible disulfide bridge.

2- Homopolymers of PLGA prepared which were also connected by bioreducible disulfide bridge.

3- Derivatisation of the hydrophilic block terminus to install a ligand for cell receptor targeting, folic acid has been chosen as targeting moeity.
4- Double emulsion technique, water in oil in water (W/O/W) was utilised to encapsulate pDNA in the matrix of the nanoparticles composed of a blend of PLGA homopolymer and PLGA-PEGMA diblock copolymer suface-functionalised with folic acid.

The synthetic strategy to prepare the diblock copolymer of PLGA-S-S-PEGMA relied on the combination of two different polymerisation techniques, ROP and ATRP. The selection of ATRP method was because many commercially available acrylic ester monomers can be polymerised with a high degree of control. Moreover, polymers prepared by ATRP techniques enable the derivatisation of the terminal polymer via well established click chemistries. This is crucial and versatile method for installing targeting moieties such as folic acid.
This approach is considered to be superior to conventional method which require to use heterobifunctional PEG in order to obtain such diblock copolymer with functionalised end-terminal.

The first key compound in the synthetic strategy was the heterobifunctional initiator (incorporated disulfide brigdge) which was used to initiate the ROP of lactide and glycolide monomers at one end using hydroxyl moiety and then polymerisation of PEG-methacrylate monomers at the other end (PLGA-S-S-PEGMA) using a bromine centre. The second key compound was the commercially available dithiodiethanol compound. This has been utilised to grow PLGA polymer from both ends simultaneously (PLGA-S-S-PLGA).

It was possible to transform the bromine at the end of the PLGA-S-S-PEGMA chains into an azide terminal groups in a single step which subsequently were used in a "click chemistry" reaction to install derivatised folic acid at the end of the PEGMA chain. The reason for using " click chemistry" was to make a versatile method to attach different molecules (targeting moieties or imaging agent or both together) derivatised with acetylene. Secondly, the "click chemistry" reaction used was very specific, compatible with various bioconjugation techniques and was efficient which allowed to obtain a very high yield of product.

The next stage was the fabrication of core-shell blended nanoparticles via double emulsion technique (previously described in chapter two of the thesis). The copolymers of PLGA-S-S-PEGMA-folate and PLGA-S-S-PLGA were mixed together in the organic solution of the double emulstion technique. The hypothesis was that blending of PLGA-PEGMA-folate in the initial steps of double emulsion technique may enhance the encapsulation efficiecy of pDNA. This may occur through stabilisation of the primary emulsion by PLGA-PEGMA-folate which is amphiphilic in nature (surfactant) and thus prevents pDNA leakage.

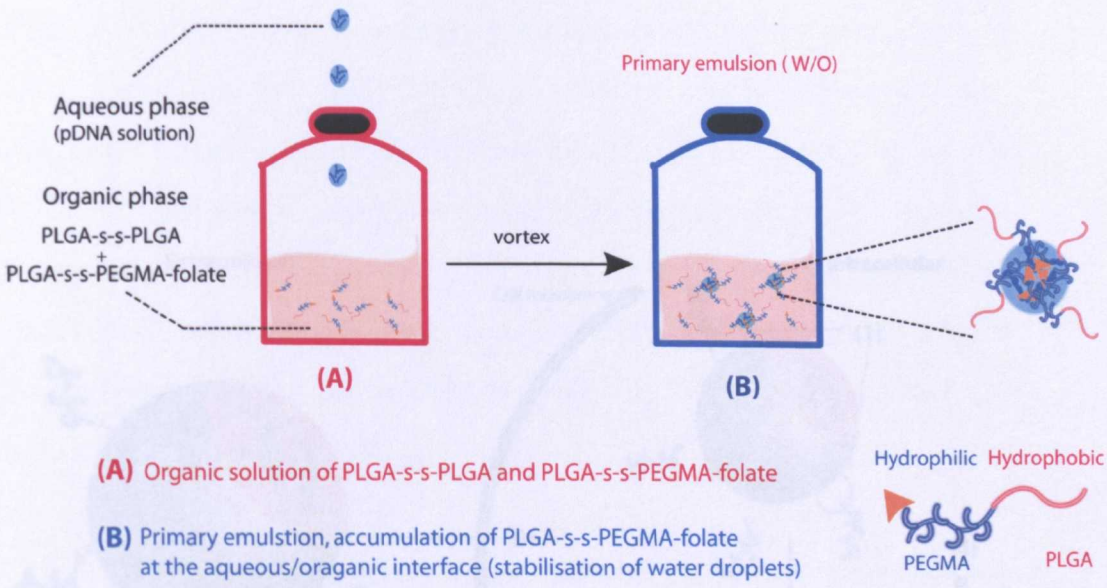

Figure 5-7. Schematic illustration of primary emulsion stabilised by PLGA-S-S-PEGMA-folate diblock copolymers.

Using double emulsion technique and blend copolymer systems made it possible to achieve higher encapsulation efficiency up to 80% of plasmid DNA. The next step was to enhance pDNA release from the nanoparticles, this is a crucial step to ensure efficient delivery of the pDNA inside the cell and this has been performed using the following strategies

1- Incorporation of disulfide bridge in PLGA-S-S-PEGMA-folate to trigger the loss of the polymer shell intracellularly (mammalian cells contain ~ 5mM glutathione concentration as reducing agent).
2- Incorporation of disulfide bridge in PLGA-S-S-PLGA polymer which mainly form the bulk matrix of the nanoparticles (i.e. disulfide bridge distrubuted throughout the matrix). This is in addition to hydrolytic degradation of PLGA polymer backbone.

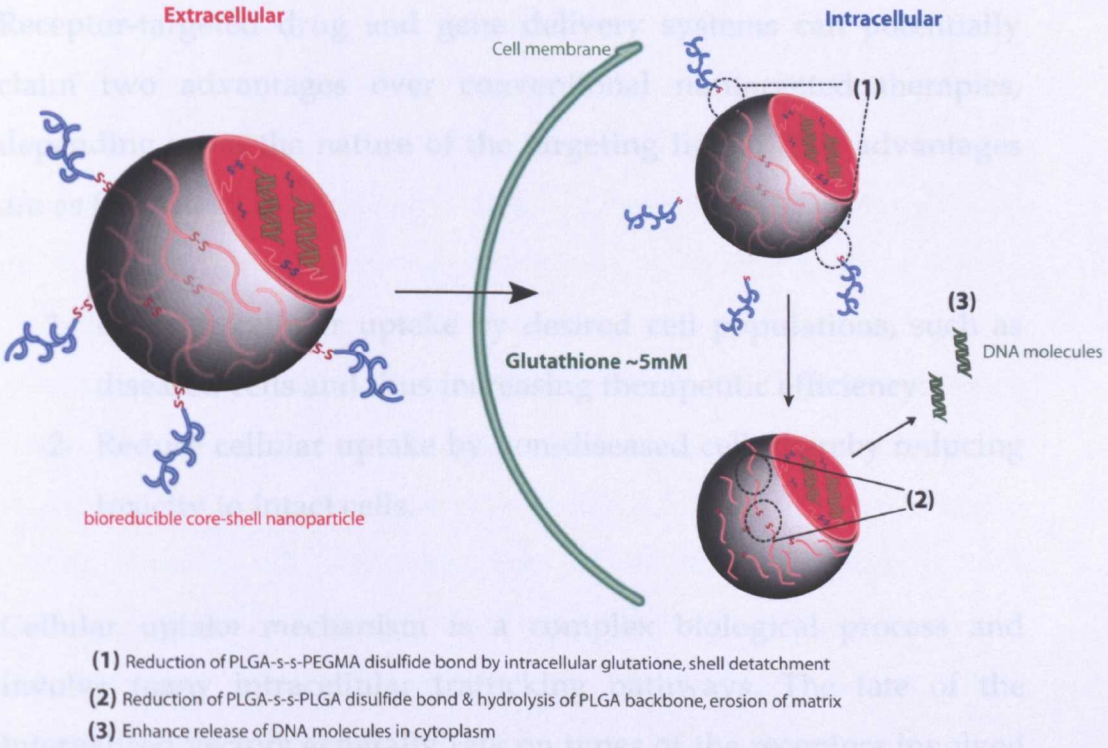

Figure 5-7. Schematic illustration of reducible core-shell nanoparticles, intracellular unpackaging in the presence of glutathione (reducing agent) to enhance the release profile of encapsulated DNA.

Once the reducible nanoparticles were internalised, the nanoparticles followed dual degradation stages. First, the presence of intracellular glutathione triggered the loss of the shell and secondly biodegradation of the particles took place via reduction of the disulfide bridges. The final degradation stages involve the hydrolytic degradation of PLGA polymer backbone which result in the erosion of the matrix and further release of the "cargo", (although in the thesis the full degradation of the PLGA was not evaluated).

So far, the concepts and strategies for construction of reducible core-shell nanoparticles with higher encapsulation efficiency and improved release profile have been discussed. The next step was to surface functionalise the nanoparticles to achieve specific targeting. Receptor-targeted drug and gene delivery systems can potentially claim two advantages over conventional nontargeted therapies, depending upon the nature of the targeting ligand. The advantages are as follow:

1- Enhance cellular uptake by desired cell populations, such as diseased cells and thus increasing therapeutic efficiency.
2- Reduce cellular uptake by non-diseased cell, thereby reducing toxicity to intact cells.

Cellular uptake mechanism is a complex biological process and involve many intracellular trafficking pathways. The fate of the internalised vectors generally rely on types of the receptors involved and related intracellular trafficking. Therefore, to ensure safe and efficient delivery of the therapeutic molecules to the targeting site, careful selection of the receptors for targeting is crucial. In the delivery of RNA and DNA molecules, the site of actions are cytoplasm and nucleus respectively. Targeting moieties such as antibodies or hormones are internalised by cells as a method of clearing the ligands from their cell surfaces (i.e. once a hormone signals through its receptor, it must be removed and destroyed to prevent continuous signaling for the life of the cell). In contrast, folic acid is taken up by the cells via the folate receptor, an essential element required for cell functions, and therefore, vectors conjugated with folic acid are not delivered to lysosomes, but rather are released into cytosol.

In addition, the significant upregulation of folate receptor on most cancer cells and high biniding afinity of folic acid have generated extensive interest in folic acid to utilise as targeting moiety for delivery of many therapeutic molecules.

In this work, folic acid was chosen as the model of targeting ligand and conjugated to the surface of reducible core-shell nanoparticles via "click chemistry". The lung cancer cell line Calu-3 was also chosen as a clinically relevant model to examine the uptake efficacy of folate functionalised nanoparticles. Owing to high binding afinity of folic acid and folic acid conjugate to its receptor, saturation of the receptor is more likely to occur. Therefore, the cellular experiment assay was carried out in a way that the amount of folic acid on the nanoparticles were progressively increased to achieve maximum cellular uptake and avoid receptor saturation. To confirm the FR mediated cellular uptake of the nanoparticles, the folate receptors on the calu-3 cell lines were saturated by adding excess amount of free folate.

5.7. Concluding Remarks

Gene therapy provides great opportunities for treating inherited and acquired diseases such as cystic fibrosis and cancer. The success of gene therapy relies on the availablity of efficient and safe delivery carriers to transport therapeutic DNA into desired cell populations. Polymeric gene delivery systems have been developed as alternatives to viral vectors for reasons of their avoidance of cytoxicity and immunogenecity. PLGA is biodegradable and biocompatible, and is an FDA approved polymer that has a significant potential for delivery of biomacromolecules such as pDNA and proteins in the form of micro and nanoparticles.

The primary limitations to PLGA particulate delivery systems includes exposing pDNA to harsh condition (high shear forces)s during nanoparticle formulations, low encapsulation efficiencies and poor release profile (burst release). Several strategies have been considered to overcome these problems, in particular, preserving the integrity of pDNA during particule formulation, among which was the addition of other biocompatible polymers to form blended particulate systems such as poloxamers and poloxamines.

In Chapter 2 of the thesis, a new blended particulate system based on biocompatible and biodegradable polymers (PLGA and Jeffamine™) has been reported with significant improvement to the encapsulation efficiency of DNA as well as the releasing profile. The data analyses for the new blended system have confirmed the success of the hypothesis and a strategy was undertaken to circumvent the aforementioned problems associated with PLGA delivery systems.

The block copolymers of PLGA-PEGMA and PEGMA-PDMAEMA have been synthesised and physically adsorbed on the new blended PLGA: Jeffamine system to provide a hydrophilic protecting shell which is crucial in preventing particle aggregation and in vivo stability.

To avoid the controversy around the in vivo stability of particles physically coated with hydrophilic polymers, in chapter three of the thesis, new strategies have been developed to formulate particles coated with hydrophilic polymers via covalent bonds. Overall it has been shown that it is possible to synthesise block co-polymers based on biodegradable PEG-methacrylate and PLGA components in a

facile one-pot procedure and with good control over block length, proportion and final molar mass.
These polymers assemble in aqueous solution into micelles that can encapsulate model drug compounds or can be processed in double emulsion techniques to encapsulate DNA molecules.

Release studies with fluorescent model drugs showed that overall release was higher with micelles formed from polymers that, at the same mass, exhibited higher micellar volume, but that release rates were lower for co-polymers with a higher proportion of hydrophobic:hydrophilic blocks. Successful uptake of micelles into 3T3 cells was shown for two block co-polymers used, polymers 5 and polymer 8.

The combination of ROP and RAFT chemistries used here to make the PLGA-PEGMA polymers offers many advantages compared to existing routes to PLGA-PEG type co-polymers for future studies of controlled release systems. The tailoring of co-monomer type and the wide variety of methacrylate and acrylate functional materials available that can be polymerised via RAFT or ATRP means that fine-tuning of drug incorporation and release should be possible, and via external or biological triggers for targeted therapies.

In Chapter 4 of the thesis, significant steps have been made toward the construction of multifunctional nanoparticles that fulfill all the requirements for a successful nonviral gene delivery system in a single carrier. Overall, the work that has been carried out was concluded as the syntheses of modular block copolymers, based on PLGA and PEG-methacrylate, assembled via combination of ROP,ATRP and click chemistry routes to generate biocompatible, cell-targeted and bioresponsive gene delivery vehicles.

Double emulsion techniques were used to encapsulate DNA with high efficiency, and uptake of the resultant nanoparticles was shown to take place in Calu-3 cells via a specific folate-receptor mechanism. The reporter gene expression studies demonstrated that selective receptor-mediated and intracellularly triggered drug delivery took place, and the ability to do this in the clinically important Calu-3 cell line offers promise for future therapeutic applications.

5.8. Future Work

The developed nanoparticulate systems seem to be rather promising and potential candidates as drug and gene delivery systems. The synthetic strategies to prepare diblock copolymers in a controlled manner and the possibility of subsequent functionalisation offer great opportunities to prepare carriers with tailored properties. Despite the success of *in vitro* studies for the developed nanoparticulate systems, *in vivo* experiments need to be carried out in order to evaluate the efficacy of the systems for clinical applications. Depending upon the desired size, biodistribution and fate of the nanocarrier, the length of the block and composition of the polymers can be tuned for specific applications. The release profile also can be improved depending on the specific properties needed. Bolus release (immediate) or prolonged release can be obtained by incorporation of different functionalities or by changing the composition of lactide to glycolide units in the PLGA polymer. Finally, it would be advantageous to incorporate more than one therapeutic agent together in a single nanoparticulate system to achieve combinatory doses in a single dosage to produce a synergistic effect such as in the case of cancer which requires combination therapies.

5.9. References

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